Integrating dark and light biohydrogen production strategies: towards the hydrogen economy

Redwood, Mark; Paterson-Beedle, Marion; Macaskie, Lynne

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Integrating dark and light bio-hydrogen production strategies: towards the hydrogen economy

Mark D. Redwood, Marion Paterson-Beedle and Lynne E. Macaskie

School of Biosciences, University of Birmingham

Correspondence should be addressed to Dr Mark D. Redwood, School of Biosciences, University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK.

Tel: +44 1214145889

Fax: +44 1214145925

e-mail: m.d.redwood@bham.ac.uk

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Abstract

Biological methods of hydrogen production are preferable to chemical methods because of the possibility to use sunlight, CO_2 and organic wastes as substrates for environmentally benign conversions, under moderate conditions. By combining different microorganisms with different capabilities, the individual strengths of each may be exploited and their weaknesses overcome. Mechanisms of bio-hydrogen production are described and strategies for their integration are discussed. Dual systems can be divided broadly into wholly light-driven systems (with microalgae/cyanobacteria as the 1st stage) and partially light-driven systems (with a dark, fermentative initial reaction). Review and evaluation of published data suggests that the latter type of system holds greater promise for industrial application. This is because the calculated land area required for a wholly light-driven dual system would be too large for either centralised (macro-) or decentralised (micro-)energy generation. The potential contribution to the hydrogen economy of partially light-driven dual systems is overviewed alongside that of other bio-fuels such as bio-methane and bio-ethanol.

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Glossary & Abbreviations

ADP: Adenosine diphospahte

Akinete: Vegetative cyanobacterial cell accumulating carbohydrate. The main component of filaments, including heterocysts

APB: Anoxygenic photosynthetic bacteria

ATP: Adenosine triphosphate

Autotrophy: Metabolism with the synthesis of carbohydrate using light and/or inorganic substrates

Av. : Average

Axenic: Pure culture containing only one type of microorganism

BOD: Biological oxygen demand; the mass of oxygen consumed by microorganisms during the oxidation of organic compounds from a sample of water

COD: Chemical oxygen demand; the mass of oxygen consumed during the chemical oxidation of organic compounds from a sample of water

CSTR: Continuously stirred tank reactor

Direct bio-photolysis: H_2 production from water; electrons liberated from H_2O by photosystem II recombine with H^+ to form H_2, catalysed by hydrogenase or nitrogenase

DF: Dark fermentation

DF-PF: dual system combining dark fermentation and photofermentation

DMFC: Direct methanol fuel cell, a type of PEM-FC using methanol fuel directly without reforming as in the indirect methanol fuel cell

dw: Dry cell weight

FHL: Formate:hydrogen lyase

Fermentation: Microbial growth mode in which ATP is generated only by substrate level phosphorylation in the absence of exogenous electron acceptors (e.g. O_2, NO_3^-, NO_2^{2-}, SO_4^{2-})

HRT: Hydraulic retention time. The total flow rate though a diluted system over its volume

Indirect bio-photolysis: H_2 production from water *via* the photosynthesis and fermentation of carbohydrates

Heterocyst: A cyanobacterial cell specialised for N_2 fixation

Heterotrophy: Microbial metabolism utilising organic carbon sources

HHV: higher heating value

Hyperthermophilic: refers to extreme thermophiles most active in the temperature range 80 – 110 ºC

LDH: Fermentative lactate dehydrogenase

Light conversion efficiency: The percentage of available light energy converted to H_2, distinct from photosynthetic efficiency (PE)

Mesophilic: Most active in the temperature range 20 – 40 ºC

NADH: Nicotinamide-adenine dinucleotide

Net energy ratio: The dimensionless ratio of the energy outputs to primary inputs for the entire operational lifetime of a system

Nitrogenase: Nitrogenase complex (reductase and nitrogenase)

PE: Photosynthetic efficiency. The percentage of photosynthetically active light energy converted to H_2. (includes only those wavelengths which interact with photopigments)

PEM-FC: Proton exchange membrane fuel cell; a type of low-temperature fuel cell considered most suitable for transport applications

PF: Photofermentation

PHB: Poly-β-hydroxybutyrate, a storage polymer

Photoheterotrophy: light-driven mode of anaerobic metabolism using organic substrates as electron donors.

Pi : Inorganic phosphate

PFL: Pyruvate:formate lyase

PFOR: Pyruvate:ferredoxin oxidoreductase

Photopigments: Light harvesting proteins

PEM-FC: Proton-exchange membrane fuel cell

Phototrophy: Microbial metabolism using light energy

Photoautotrophy: Microbial metabolism using light energy for the synthesis of carbon sources

PNS bacteria: Purple non-sulfur bacteria

PSI: Photosystem I

PSII: Photosystem II

Reserve: The amount of a resource in place (e.g. oil in the ground) that is economically recoverable

SOFC: Solid oxide fuel cell, a high temperature alkaline fuel cell

SOT medium: Growth medium for cyanobacteria containing salts and trace elements but no carbon source

Thermophilic: Most active in the temperature range 40 – 60 ºC

UASB: Upstream anaerobic sludge blanket reactor.

1: Bio-fuels for sustainable energy production

An estimated 45 % of the identified world oil reserves has been combusted and atmospheric CO_2 has increased by 20 % since 1900 (Holmes & Jones 2003; Keeling & Whorf 2005). Conservative estimates suggest that demand for oil will outstrip supply by 2050 (Holmes & Jones 2003), while the Stern Review (Stern 2006) highlights the urgent need for reduction in greenhouse gas emissions. A sustainable energy economy is needed and this will require a different fuel; one that is not limited in supply and whose use is environmentally benign. Hydrogen is now recognised as a key energy vector in the future energy economy. H_2 storage technology and fuel cell efficiency continue to receive urgent attention and have advanced sufficiently for transport applications to approach commercial viability. For example, BMW's fleet of 100 "Hydrogen 7" cars, each having a material value of $500,000, is now available for promotional purposes (carsguide.com.au, Nov 2006). A prototype H_2-powered boat is also opening the way to economic transport *via* inland waterways (Bevan et al. 2007). Whereas the use of H_2 in transport applications is dependent upon such emerging technologies, its use in stationary applications, for electricity supply, is limited primarily by H_2 availability.

Biological approaches could contribute to large-scale H_2 production as various microorganisms can produce H_2 under moderate conditions from readily available, renewable substrates, making biological strategies potentially competitive with chemical process such as reforming and gasification. Bio-hydrogen processes are 'CO_2-neutral', being fuelled by carbohydrates originating from photosynthetic fixation of CO_2. Furthermore, bio-H_2 is free of CO and H_2S (both catalyst poisons) and requires no treatment before use in fuel cells for electricity generation (Macaskie et al. 2005).

Suitable feeds for bio-hydrogen generation processes can be found in agricultural residues (Nath & Das 2003; Hawkes et al. 2008), food wastes (Franchi et al. 2004; Karlsson et al. 2008) and effluents from industrial processes such as refining sugar (Yetis et al. 2000; Ren et al. 2006), distilling alcohol (Sasikala et al. 1992), olive processing (Eroğlu et al. 2004), producing cheese (Davila-Vazquez et al. 2008b) and producing tofu (Zhu et al. 1995; 2002). Hence, microbial processes could be employed to remediate wastes while simultaneously producing H_2 with the dual economic benefit of energy production and savings in the cost of waste disposal. In the UK the majority of waste is disposed by land-filling and the related environmental damage is being recognised in financial terms *via* landfill tax; which is paid additionally to normal landfill fees. In 2008, UK landfill fees were in the range GBP 11-40/tonne, to which landfill tax added GBP 24/tonne increasing by £8/year (WRAP Gate Fees Report, 2008, www.wrap.org.uk/marketknowledge). The avoidance of waste disposal costs is, therefore, anticipated to be an important economic driver in the start-up of bioenergy processes.

The capability for H_2 formation is widespread among microorganisms, but only a few have been investigated with a focus on bio-hydrogen production. In particular, photosynthetic microorganisms such as microalgae, cyanobacteria and purple bacteria are of interest, along with various dark fermentations. Each of these candidates represents a potential method in its own right, but it has long been recognised that multi-organism systems, combining the capabilities of different species would be required to realise the maximum potential for bio-hydrogen production (Rocha et al. 2001; Wakayama & Miyake 2001; de Vrije & Claassen 2003; Nath et al. 2005; Basak & Das 2007). Several examples of dual systems are illustrated in Table 1. The purpose of this review is to advance the state of knowledge by comparing the successes of diverse strategies, relating them to the methods employed, evaluating the potential for energy generation and by highlighting potential problems.

2: The use of microorganisms for H_2 production

This work does not attempt to review microbial hydrogen production (for reviews see Vignais et al. 1985; Blankenship et al. 1995; Sasikala et al. 1995; Nandi & Sengupta 1998; Claassen et al. 1999; Das & Veziroglu 2001; Hallenbeck & Benemann 2002; Nath & Das 2004a, 2004b; Bae et al. 2005; Dutta et al.

2005; Hawkes et al. 2007; Tsygankov 2007), but provides a summary of those organisms which have been studied expressly for the purpose of H_2 production. Combining different organisms in multi-organism strategies creates the possibility to exploit the most useful facets of different metabolisms. In order to evaluate multi-organism strategies, the broad metabolic mechanisms are first considered individually.

2.1: Photobiological hydrogen production

Many microorganisms have evolved the capacity to harness solar energy for growth and several types of photosynthetic microorganism are potentially useful for bio-hydrogen production. Artificial light sources are often used as models for future applications with sunlight, but solar bio-hydrogen projects have been successful (see below) despite the diurnal and seasonal variations in light intensity.

2.1.1: Photoautotrophic microorganisms

Photoautotrophs produce H_2 by two distinct mechanisms: "direct photolysis" and "indirect photolysis" which can both occur in the same organism (Figure 1). Like higher plants, microalgae (green unicellular algae) and cyanobacteria (previously called blue-green algae) have two photosystems (Photosystems I and II: PSI and PSII), which produce H_2 by "direct photolysis" in which water is decomposed to H_2 and O_2 (oxygenic photosynthesis). The dual photosystems split water, reducing electron carriers and exporting protons to generate a proton gradient for ATP generation (Miyake et al. 1999).

Hydrogenase and nitrogenase enzymes are found in cyanobacteria, but (as in purple bacteria, see later) self-sustained H_2 formation in the light results from the activity of nitrogenase, which consumes ATP and re-oxidises electron carriers. In microalgae a hydrogenase performs the reduction of $2H^+$ to H_2 without any ATP requirement. In "indirect photolysis", CO_2 is fixed *via* the Calvin cycle to synthesise simple sugars which are then accumulated as polycarbohydrates (starch in microalgae and glycogen in cyanobacteria). Stored carbohydrates can be metabolised fermentatively (section 2.2) to generate H_2 indirectly.

In direct photolysis H_2 production is limited due to the inhibition of hydrogenase and nitrogenase by oxygen generated from water. In contrast indirect photolysis separates the production of O_2 and H_2 spatially (into compartments) or temporally (into aerobic and anaerobic phases) as described below (Levin et al. 2004a) and is therefore sustainable. For example, continuous H_2 production was achieved for up to 1 month using *Anabaena cylindrica*, when O_2 was artificially removed from the culture by purging with argon gas (Madamwar et al. 2000).

Cyanobacteria are divided into non nitrogen-fixing varieties (e.g. *Synechococcus* spp.) which form only one kind of cell (akinetes) and nitrogen-fixing varieties (e.g. *Nostoc, Anabaena* spp.), which form akinetes and also heterocysts arranged into filaments - chains of cells connected by channels for the exchange of nutrients (Tsygankov 2007). Heterocysts differ from akinetes due to the absence of O_2 generation by PSII, the increased rate of O_2 consumption by respiration, the presence of a thick envelope to limit the ingress of environmental O_2 and the expression of nitrogenase to fix N_2 as NH_4^+, supporting the growth of the adjacent akinetes (Madamwar et al. 2000; Tamagnini et al. 2002). Heterocystous cyanobacteria separate H_2 production and O_2 production spatially (by compartmentalisation) accumulating glycogen in the vegetative akinetes and fermenting it to produce H_2 in the anaerobic heterocysts. Non N_2-fixing cyanobacteria and microalgae separate H_2 production and O_2 production temporally, producing H_2 by the dark anaerobic fermentation of photosynthesised carbohydrates (Carrieri et al. 2008). Upon transition to darkness, the generation of O_2 by the photosystem ceases and residual O_2 is consumed by respiration enabling H_2 production (Tsygankov 2007).

The capacity for self-sustained aerobic H_2 production in the light (i.e. without the artificial removal of O_2), is an advantageous property of heterocystous cyanobacteria (e.g. *Anabaena* spp.) achieving maximum H_2 production rates of *ca.* 100 μmol H_2/mg chlorophyll *a*/h, with light conversion efficiencies of

up to 3.9 % (proportion of absorbed light energy converted to H_2) (Dutta et al. 2005; Yoon et al. 2006; Sakurai & Masukawa 2007). Rates were increased 3-7 fold in *Anabaena* mutants deficient in uptake hydrogenase activity (Borodin et al. 2000; Happe et al. 2000; Masukawa et al. 2002; Yoshino et al. 2007) and this strategy was applied in outdoor culture. However, the maximum light conversion efficiency was only 0.1 %, which has implications for the large scale application of this approach (Lindblad et al. 2002; Tsygankov et al. 2002).

Unicellular cyanobacteria have also been studied with a view to dark fermentative H_2 production, being unsuitable for photoproduction of H_2 due to their high (competing) uptake hydrogenase activity in the light (Troshina et al. 2002). However, a mutant of *Synechocystis* deficient in uptake hydrogenase activity photoproduced H_2 at a rate of 6 μmol H_2/mg chlorophyll *a*/h (2 mL/L/h) (Cournac et al. 2004).

Like unicellular cyanobacteria, microalgae were originally studied for dark H_2 production by indirect photolysis (Miyamoto et al. 1987). The isolation of *Chlamydomonas* spp. MGA161 having a high rate of H_2 photoproduction (6 mmol H_2/g chlorophyll *a*/h), high starch accumulation (18 % w/w) and unusually rapid and efficient dark fermentation (2 mol H_2/mol starch-glucose) prompted the study of a dual system (Miura et al. 1986) (Table 1).

The extent of metabolic engineering success in microalgae lags behind that of cyanobacteria due to the greater difficulty of eukaryotic genetic engineering. Work is ongoing to improve light conversion efficiency through the truncation of light-harvesting antenna complexes, an approach already proven using PNS bacteria (see below, section 2.1.2) (Akkerman et al. 2002; Polle et al. 2002). Other approaches are to study and develop O_2-tolerant hydrogenases (Ghirardi et al. 2005; Tosatto et al. 2008) and to express clostridial hydrogenase in non-heterocystous cyanobacteria, the aim being to engineer the rapid and ATP-independent (hydrogenase-mediated) H_2 production by direct photolysis in a fast-growing host organism, possibly overcoming O_2-inhibition through strong expression (Miyake et al. 1999).

A relatively new method to increase rates of H_2 production by direct photolysis is nutrient deprivation. Under conditions of sulfate-limitation the iron-sulfur clusters of PSII subunits cannot be maintained and PSII activity is inhibited (Wykoff et al. 1998). The rate of O_2 production decreases, while the respiration rate remains high and establishes anoxia, which permits hydrogenase and/or nitrogenase expression. The result is sustained H_2 production *via* direct photolysis. The technique was pioneered using microalgae (Benemann 1996; Jo et al. 2006; Laurinavichene et al. 2006) and has been recently extended to cyanobacteria (Antal & Lindblad 2005).

2.1.2: Photoheterotrophs

Purple non-sulfur (PNS) bacteria are anoxygenic photosynthetic bacteria which, unlike the purple and green sulfur bacteria, do not produce H_2S (a powerful catalyst poison) and the off-gas is typically > 90 % H_2, hence it is suitable for use in PEM-fuel cells without purification (Nakada et al. 1995).

PNS bacteria produce H_2 under photoheterotrophic conditions (light, anaerobiosis, organic electron donor) although they are metabolic generalists capable of autotrophic and heterotrophic growth. The best-studied species belong to the genera *Rhodobacter, Rhodopseudomonas* and *Rhodospirillum*.

H_2 is produced by the nitrogenase enzyme, which is active anaerobically under nitrogen limitation (Vignais et al. 1985). In the absence of N_2 the production of H_2 occurs according to equation 1.

$$2e^- + 2H^+ + 4MgATP \rightarrow H_2 + 4MgADP + 4P_i \quad \text{(Koku et al. 2002)} \qquad (1).$$

In this respect the reaction serves to dissipate excess ATP and reducing power where growth is nitrogen-limited. The nitrogenase complex must be saturated with ATP and also NADH for optimal

activity, hence H_2 photoproduction occurs most rapidly under saturating light intensity at the expense of organic electron donors.

Nitrogenase activity is strictly a facet of anaerobic metabolism since O_2 damages the photopigments needed to maintain ATP flux for nitrogenase activity and nitrogenase expression is strongly inhibited by oxygen (Koku et al. 2002). Sustained H_2 photoproduction is possible as the single photosystem (PSI) of these organisms does not generate O_2 (this is termed anoxygenic photosynthesis) and continuous H_2-producing cultures have been operated for up to several months (Liessens & Verstraete 1986; Weetall et al. 1989; Eroğlu et al. 1997; Hassan et al. 1997; Fascetti et al. 1998; Tsygankov et al. 1998; Yokoi et al. 2001; Franchi et al. 2004; Shi & Yu 2006).

Rocha et al. (2001) analysed a large number of reports, indicating that the efficiency of light conversion to H_2 is variable for PNS bacteria, the average value being *ca.* 4 %. The theoretical maximum photosynthetic efficiency is considered to exceed 10 % (Akkerman et al. 2002) but the photosystems of PNS bacteria saturate at low light intensity, leading to low light conversion efficiency under high light intensity, e.g. in solar photobioreactors (Wakayama & Miyake 2002). PNS bacteria are adapted to photosynthesis at low light intensities, requiring large light harvesting complexes to capture diffuse light energy and conduct it into the reaction centre. Light conversion efficiency may be improved beyond 10 % by genetic manipulation to reduce the size of light-harvesting antennae, thereby increasing the saturating light intensity (Table 2). This would allow efficient H_2 production at higher light intensities, by deeper or denser cultures (Miyake et al. 1999; Vasilyeva et al. 1999; Kondo et al. 2002; Kim et al. 2004; Kondo et al. 2006; Kim et al. 2006a) (Table 2).

Nitrogenase-mediated H_2 formation is irreversible (Hillmer & Gest 1977b), which is an advantageous property in relation to reversible hydrogenase-mediated H_2 production, which is inhibited under high partial pressure of H_2 (Valdez-Vazquez et al. 2006). However, in PNS bacteria, uptake hydrogenase activity can detract from H_2 yields (Sasikala et al. 1990), prompting the development of uptake hydrogenase deficient mutants with up to 70 % increased H_2 production efficiency (Willison et al. 1984; Jahn et al. 1994; Worin et al. 1996; Öztürk et al. 2006) (Table 2).

Nitrogenase re-oxidises electron carriers to reduce $2H^+$ to H_2 and any other reductive processes ('electron sinks') can compete with and detract from H_2 production. The formation of the carbon storage polymer poly-β-hydroxybutyrate (PHB) from acetate is such a competing reductive reaction (equation 2) (Vincenzini et al. 1997; Khatipov et al. 1998).

$$9n\ CH_3COOH \rightarrow 4(CH_3CH_2CHCOO^-)_n + 2n\ CO_2 + 6n\ H_2O \text{ (Tabita 1995)} \qquad (2).$$

Mutagenesis of the PHB synthase gene yielded PHB deficient mutants, which were capable of H_2 production under conditions that would normally favour PHB synthesis (Hustede et al. 1993) (Table 2). In recent studies, double mutants lacking uptake hydrogenase and also PHB synthase produced H_2 at up to 2.5-fold higher rates compared to the parent strain (Lee et al. 2002; Kim et al. 2006b), while in a separate study a similar double mutant sustained H_2 production for over 45 days, while the wild-type ceased H_2 production after 10 days (Franchi et al. 2004).

Numerous simple organic molecules serve as suitable electron donors for PNS bacteria, including common fermentation products such lactate, acetate, butyrate, propionate and succinate (Hillmer & Gest 1977a, 1977b), alcohols such as ethanol and propanol (Fuji et al. 1987) and other substrates such as aromatic acids (e.g. cinnamate, benzoate) (Sasikala et al. 1994b; Fissler et al. 1995). Substrate range is strain-specific (Tao et al. 2008) and the biochemical pathways of assimilation are uncertain for many of these substrates, with the exception of acetate (a common fermentation product). In most bacteria acetate is assimilated using the glyoxylate cycle, but a diverse group of microorganisms (including *Rhodobacter sphaeroides* and *Rhodospirillum rubrum)* lacks the key glyoxylate cycle enzyme, isocitrate lyase, while rapidly assimilating acetate. An alternative citramalate cycle is now thought to operate in these species (Ivanovskii et al. 1997; Filatova et al. 2005a; 2005b). The distinction is important in the context of H_2 production as species lacking

the glyoxylate shunt generally require the availability (not the addition) of CO_2 during H_2 production from acetate, with the exception of *R. sphaeroides* which has a high capacity for acetate consumption (and hence CO_2 production) compared to other PNS bacteria and also has a thick capsule obstructing the diffusion of produced CO_2 (Table 2). Some uncertainty remains over the suitability of ethanol, a common fermentation product, as an electron donor for photoproduction of H_2. A *Rhodopseudomonas* species produced H_2 at the expense of various alcohols (Fuji et al. 1987) and ethanol was consumed simultaneously with acetate by *Rhodobium marinum* at *ca.* 50 % the rate of acetate, although the initial concentration of ethanol was *ca.* 25 % that of acetate (Ike et al. 2001). Ethanol was rapidly removed from an *Escherichia coli* fermentation effluent by *R. sphaeroides* O.U.001 after a delay of 96 h, although the induction of ethanol-utilising enzymes was not monitored (Redwood & Macaskie 2006). Hence, it is plausible that other PNS bacteria would be capable of ethanol utilisation after an adaptation period.

PNS bacteria have significant potential for industrial application as mixed cultures can be maintained for extended periods (Liessens & Verstraete 1986; Ko & Noike 2002; Fang et al. 2005; Ying Li et al. 2008), industrial waste streams can make suitable feeds for the photoproduction of H_2 (Thangaraj & Kulandaivelu 1994; Fascetti et al. 1998; Yiğit et al. 1999; Zhu et al. 1999a; Yetis et al. 2000), larger scale photobioreactors are under development (Hoekema et al. 2002; Hoekema et al. 2006l; Claassen & de Vrije 2007) and outdoor projects using sunlight have been successful (Wakayama et al. 2000; Wakayama & Miyake 2002; Eroğlu et al. 2008).

The foremost limitation with PNS bacteria is the incompatibility of nitrogenase activity and the presence of NH_4^+. Waste streams can only be used for H_2 production if they are of high C/N ratio and many reports of this application are available (Sasikala et al. 1992; Turkarslan et al. 1997; Tsygankov et al. 1998; Yiğit et al. 1999; Yetis et al. 2000; Eroğlu et al. 2004; Fang et al. 2005). Low C/N waste streams have been applied successfully for the purposes of biomass production and effluent remediation (Ensign 1977; Hassan et al. 1997; Cornet et al. 2003; Yun & Ohta 2005). H_2 production using low C/N feeds has been accomplished by the use of immobilisation matrices which exclude cations such as NH_4^+ (Zhu et al. 1999b; Zhu et al. 2001) and the development of nitrogenase-derepressed strains (Wall & Gest 1979; Zinchenko et al. 1991; Yagi et al. 1994; Zinchenko et al. 1997) (Table 2). These approaches were not, however, tested at pilot-scale or in continuous culture and issues such as the economic viability of immobilisation and the long-term stability of nitrogenase-derepressed strains remain to be addressed.

PNS bacteria are capable, therefore, of efficient conversion of organic acids to H_2, providing a potentially applicable method for the remediation of wastes rich in organic acids, alcohols or aromatics. With the exception of unusual strains (Macler et al. 1979; Macler & Bassham 1988; Oh et al. 2004), PNS bacteria lack the capacity for the efficient conversion of sugars to H_2 and for this application a dark fermentation is the method of choice.

2.2 Dark Hydrogen fermentation

Large quantities of simple and complex carbohydrates are available as agricultural and food processing residues (Easterly & Burnham 1996; Filho & Badr 2004; Haq & Easterly 2006; Mabee et al. 2006; Levin et al. 2007). Fermentative bacteria represent a promising means not only to reclaim energy from these wastes in the form of hydrogen but also to utilise the wastes as resources, a particularly valuable attribute given the escalating cost of landfill (Bartelings et al. 2005). Indeed, it was calculated that the savings in landfill tax would be the main economic driver, outweighing the value of the energy produced *via* dark-fermentative production of H_2 from confectionery waste (Macaskie 2004).

The anaerobic degradation of organic matter by heterotrophic microorganisms can liberate H_2 at high rates, depending on the particular organisms and conditions. Fermentation generates energy solely through substrate level phosphorylation. Substrates are converted to reduced compounds, which are excreted as waste products and the ATP yield is low, in comparison to respiration. The formation of relatively reduced organic molecules is an integral part of all dark fermentations and some of these molecules (e.g. acetate) can

inhibit H_2 production if allowed to accumulate (Roe et al. 1998; Kirkpatrick et al. 2001; Van Ginkel & Logan 2005).

In a minority of fermentative microorganisms (e.g. *Klebsiella* spp.) H_2 production is primarily mediated by nitrogenase (Vignais et al. 2001) but due to the high ATP requirement and low turnover rate of nitrogenase, the theoretical H_2 yield is only 0.5 mol H_2/mol hexose (Wakayama & Miyake 2001). Without the contribution of light energy through photosynthesis, hydrogenase is preferred for H_2 production due to its higher rate of turnover and lower metabolic cost. The highest fermentative H_2 yields have been achieved using clostridia, other enteric bacteria and hyperthermophiles (see reviews: Hallenbeck 2005; Davila-Vazquez et al. 2008a).

H_2 fermentations are restricted by the Thauer limit. Thermodynamically, no more than 4 mol H_2 can be produced from 1 mol hexose because substrate level phosphorylation must produce whole numbers of ATP and the yield of ATP from glucose must be at least 1 mol/mol for the cell to survive (Thauer 1977). However, microbial fermentation typically generates more than 1 mol ATP and less than 4 mol H_2/mol hexose, quantities that vary according to the metabolic system and conditions, as described below.

2.2.1 Axenic dark fermentations

Axenic cultures (pure cultures containing clonal microbial populations) have been used in the majority of fermentation research, creating a wealth of information regarding model organisms and the understanding of their fermentative metabolism has facilitated and rationalised the optimisation of conditions for H_2 production.

Dark fermentations are united by the initial glycolytic generation of ATP, NADH and pyruvate. Three enzymes compete for pyruvate: pyruvate:ferredoxin oxidoreductase (PFOR), pyruvate:formate lyase (PFL) and the fermentative lactate dehydrogenase (LDH). The realised H_2 yield is dependent upon the fate of pyruvate, which differs among species due to varying activities of PFL, PFOR and LDH, of which one or more may be present (Figure 2).

Mixed-acid fermentation, in which the key enzymes are PFL and the formate:hydrogen lyase (FHL) complex (comprising a specific formate dehydrogenase and hydrogenase) (Figure 2B), is performed by facultative anaerobes such as *E. coli*. PFL converts pyruvate to acetyl-CoA and formate, which is cleaved to H_2 and CO_2 by FHL, while acetyl-CoA is divided between the formation of acetate (which generates ATP) and the formation of ethanol (which oxidises NADH to regenerate NAD). PFOR is expressed constitutively to a low level (Knappe 1987), but since H_2 is entirely formate-derived (Ordal & Halvorson 1939) PFOR is not thought to be involved in H_2 production in *E. coli*.

Ideally, mixed-acid fermentation yields 2 mol H_2/mol glucose (Figure 2B), but in batch mode a yield of *ca.* 50 % of this is usually obtained due to diversion of pyruvate into lactate formation. The latter can be suppressed by control of culture conditions or through metabolic engineering (Sode et al. 1999; 2001). While the cleavage of formate is irreversible, H_2 recycling is an issue, as suggested by the observation of 37 % increased H_2 yield in Hyd-2 mutants of *E. coli* (Redwood et al. 2007c). The rate of H_2 formation was also increased through the increased expression of FHL (Penfold et al. 2003; Yoshida et al. 2005).

Facultative anaerobes of the related genus *Enterobacter* also produce H_2 from formate but analysis of the fermentation balance implicated the simultaneous activity of the NADH pathway (Tanisho & Ishiwata 1995; Tanisho et al. 1998; Kurokawa & Shigeharu 2005), in which the regeneration of NAD^+ is coupled to the reduction of ferredoxin by NADH:ferredoxin oxidoreductase (NFOR). Reduced ferredoxin subsequently transfers electrons onto H^+ to produce additional H_2. The NADH pathway (operating simultaneously with PFL/FHL) could theoretically achieve the Thauer limit (4 mol H_2/mol glucose). However both electron transfer reactions (from NADH onto oxidised ferredoxin and from reduced ferredoxin onto H^+) are reversible and neither would be considered electrochemically feasible under standard conditions: i.e. the standard electrode potentials of the NAD and ferredoxin half-cells (-320 mV and -400 mV, respectively) are more positive than that of the H^+ half-cell (-414 mV) (McCormick 1998). A very low H_2 partial pressure

(pH_2) (theoretically <60 Pa or <0.0006 bar) is required to drive this reaction forwards and H_2 yields exceeding 2 mol H_2/mol glucose were obtained only under vacuum or with continuous gas purging to strip away H_2 (Park et al. 2005). Indeed, a maximum yield of 3.9 mol H_2/mol glucose was reported using *E. cloacae* under a vacuum of 330 torr (equivalent to 0.44 bar or 44 kPa) (Mandal et al. 2006).

Clostridia also use the NADH pathway. In this case PFOR cleaves pyruvate to acetyl-CoA and CO_2, transferring electrons to ferredoxin, which is coupled to a reversible hydrogenase to produce H_2. In this situation, all H_2 is produced by a single reversible reaction and it is even more important to maintain a low pH_2 (Kataoka et al. 1997; Mizuno et al. 2000). Advances in gas separation technology may permit a purge-gas recycle system to remove the need for large quantities of inert, anaerobic purge gas for H_2 removal (Nielsen et al. 2001; Liang et al. 2002; Teplyakov et al. 2002).

A positive pressure (*ca.* 1-1.5 bar at 25 °C) is needed for H_2 uptake by metal hydride H_2-stores (Züttel 2004). Therefore, to charge a H_2 store directly from a fermentation culture (without intermediary gas-pressurisation) would require an organism capable of sustaining H_2 production under high pH_2. This would require the absence of biological H_2 recycling and would preclude a reversible H_2-producing system, such as the NADH pathway occurring in enteric bacteria and clostridia, but may be possible using an uptake hydrogenase mutant of *E. coli* in which the FHL complex (involving hydrogenase-3) performs the irreversible oxidation of formate to form H_2 (and CO_2). However, a degree of reversibility is a common property of hydrogenases (Van Haaster et al. 2005) and although hydrogenase-3 has no uptake role during fermentation (Redwood et al. 2007c), it is known to operate reversibly when coupled to redox dyes (e.g. Sauter et al. 1992). Therefore, the latter strategy may tolerate a higher pH_2, but regardless of the organism employed, a pressurisation step would be advantageous between the fermentation and the H_2-store.

Various *Clostridium* spp. have been investigated for bio-hydrogen production (Collet et al. 2004), of which *C. butyricum* is perhaps the best known. Like *E. coli* and *E. aerogenes*, this organism is mesophilic but unlike them, it is a strict anaerobe. Hence, clostridial growth media are usually supplemented with a reducing agent to ensure anaerobiosis. Alternatively, a facultative aerobe, added to the H_2-production culture, was effective as an O_2-scavenger (Yokoi et al. 2001).

The H_2 yield from *C. butyricum* could in theory reach 4 mol H_2/mol hexose (Figure 2C) although a detailed metabolic analysis of *C. butyricum* calculated a maximum of 3.26 mol H_2/mol hexose (Chen et al. 2006) and practical yields obtained using clostridia rarely exceed 2 mol H_2/mol hexose (Collet et al. 2004; Ferchichi et al. 2005).

The clostridial species selected for H_2 production produce acetate and butyrate rather than propionate but they sporulate in response to environmental stresses such as heat or nutrient depletion, hence, the feeding regimes used in continuous culture are designed to maintain excessive nutrient concentrations to minimise sporulation (Hawkes et al. 2002). Asporogenic mutants have proved advantageous in ethanol production from cellulose, but have not yet been applied to H_2 production (Taillez et al. 1983). Whereas mesophilic clostridia sporulate as temperature increases, certain clostridial species are moderately thermophilic. For example, *C. thermolacticum* prefers to grow at 54 °C (Collet et al. 2004). Recently hyperthermophiles, which live and produce H_2 at temperatures above 60 °C have been studied. Little biochemical information is yet available (e.g. de Vrije et al. 2007) but it seems that hyperthermophiles are capable of H_2 production with higher yields than mesophiles (Hallenbeck 2005). For example, a yield of 2.8 mol H_2/mol glucose was reported for *Thermotoga elfii* and 3.2-3.7 mol/mol for *Caldicellulosiruptor saccharolyticus* (Van Niel et al. 2002; Kadar et al. 2004; de Vrije et al. 2007). Observations support the connection of H_2 production with the hydrogenase-linked oxidation of electron carriers (as in clostridia), rather than the decomposition of formate (as in enteric bacteria). A pH_2 of 10-20 kPa (0.1-0.2 bar) induced a metabolic shift to inhibit H_2 production in *C. saccharolyticus* (Van Niel et al. 2003) and a limiting H_2 pressure of 20 kPa (0.2 bar) was reported for a mixed hyperthermophilic culture (Van Groenestijn et al. 2002), while formate was not decomposed by *Thermotoga neapolitana* (Van Ooteghem et al. 2004).

The necessity of growth on solid media for molecular work (i.e. at temperatures lower than the melting point of agar), makes hyperthermophiles less readily amenable to genetic engineering, although alternative solid media such as gelrite are available (Van Ooteghem et al. 2004). Thermophilic cultures are

resistant to overgrowth by mesophilic contaminants but although an economic analysis is not available, the energetic costs associated with maintaining 70 ºC may mitigate against large-scale application.

Several mesophilic and thermophilic clostridia and hyperthermophiles have the capacity to utilise complex carbohydrates such as cellulose and starch, a valuable property widening the potential for the use of industrial waste streams and agricultural residues as feeds. For example, *T. neopolitana* can utilise dextrins (Van Ooteghem et al. 2004) and *C. thermocellum* produced 1.6 mol H_2/mol hexose from delignified wood (Levin et al. 2006). Enteric bacteria generally lack the ability to metabolise complex carbohydrates although the necessary genes can be introduced in the case of *E. coli* (Dien et al. 2000).

2.2.2 Mixed dark fermentations

The use of mixed cultures offers practical advantages over the use of pure cultures, such as the use of feedstocks without pre-treatment or sterilisation and is already a proven, commercially available technology (Kyazze et al. 2007). Inocula for H_2 production can be obtained from soil, compost or anaerobic digestion sludge (Hawkes et al. 2002; 2007). H_2 was produced from sucrose using sewage sludge microflora with a yield of 1.7 mol H_2/mol hexose (Lin & Lay 2005) and from food processing effluent using a heat-treated (2 h, 104 °C) sludge inoculum with typical yields of 0.2-0.87 mol H_2/mol hexose (Oh & Logan 2005). Rice slurry was fermented by a heat-treated (30 min, 100 °C) clostridial community to produce up to 2.5 mol H_2/mol hexose (Fang et al. 2006). Paper sludge and cellulose powder were rapidly degraded by mesophilic anaerobic consortia, producing mixtures of CH_4 and H_2 (Ueno et al. 1995; Valdez-Vazquez et al. 2005).

For mixed cultures there is a tendency towards lower rates and yields of H_2 production because non H_2-producing organisms (e.g. methanogens and sulfate-reducers) consume a proportion of the substrate and perform H_2 uptake using H_2 as an electron donor. Furthermore, H_2S (the product of dissimilatory sulfate reduction) is a potent catalyst poison requiring removal if the bio-hydrogen is intended for use in a fuel cell. Hence, for the efficient production of clean H_2, the microbial population must be controlled to some degree in order to select for H_2-producers. Methanogens can be suppressed by the addition of chemical inhibitors or by operating continuous cultures at low pH or HRT (Hawkes et al. 2007). The microbial population is often manipulated by applying pre-selection on the inoculum (Valdez-Vazquez et al. 2005). A widely adopted strategy is to select for spore-forming clostridia using a heat-treated inoculum and where this can be achieved the properties of clostridial fermentation (above) are predominantly applicable (Kim et al. 2004; Van Ginkel & Logan 2005). However, heat treatment also eliminates non-sporulating H_2-producers (e.g. *Enterobacter* spp.) and selects for spore-forming H_2-consumers (e.g. some acetogens) (Kraemer & Bagley 2007). The metabolic switch from H_2 production to solventogenesis can be avoided by the intermittent release of headspace pressure and N_2 purging (Valdez-Vazquez et al. 2006).

3: Hybrid hydrogen

As reviewed above, no single-stage system has been shown to produce H_2 beyond 4 mol H_2/mol hexose. Current research focuses on the possible use of two-component systems *via* a variety of strategies (Table 1). These dual systems are united by the conversion of carbohydrates into organic acids in the 1st stage (which may be mesophilic or thermophilic and may not necessarily produce H_2), followed by the conversion of fermentation products into H_2 in the 2nd stage (Figure 3). In some examples, algae or cyanobacteria initially photosynthesise carbohydrates, which are then fermented by the same organisms, while other systems use carbohydrates as the primary feed, either as artificial solutions or in the form of wastes or algal biomass.

3.1 Techniques for connecting the components of a dual system

Alongside the choice of organisms, dual systems have been implemented through a variety of strategies. The nature of the bridge connecting these two stages is a key part of the operational strategy affecting the overall productivity of the system. The simplest approach constitutes a co-culture in which different organisms are in direct contact and act simultaneously, under the same conditions (Figure 3A). However, a compromise needs to be sought between the optimal requirements of each microbial component. While increasing the complexity and cost, sequential reactors permit the operator to maintain different conditions in separate parts of the dual system, allowing a combination of organisms, which may not be compatible in co-culture (Figure 3B). For example, (wild-type) microalgae/cyanobacteria and PNS bacteria were not compatible in co-culture since the photosynthetic generation of oxygen inhibited nitrogenase-mediated H_2 production by the PNS bacteria (Miyamoto et al. 1987; Weetall et al. 1989). Further, sequential reactor systems can be potentially more effective as either component can be optimised without compromise to the other and may be preferred even for 'compatible' combinations of organisms.

Sequential reactors require some method to transport fermentation products from the 1st reactor to the 2nd (while retaining biomass), which presents an engineering challenge for future scale-up operations. The simplest and most common method is 'batch-transfer' in which spent medium is transferred between reactors in batches. Centrifugation followed by filtration or autoclaving is usually performed to generate a clear, sterile feed for the 2nd stage (e.g. Yokoi et al. 2002; Redwood & Macaskie 2006). For large-scale application, continuous processes are generally preferred over batch systems. Fermentation products could potentially be transferred continuously through the use of bi-phasic solvent extraction, by continuous centrifugation or by membrane systems (Banik et al. 2003; Emanuelsson et al. 2003; Splendiani et al. 2003), but these techniques have yet to be applied in a H_2-producing system.

3.2 Comparing diverse strategies

As explained above, the two components of a dual system may be bridged in several ways. To add to the difficulty of comparison, either part of the dual system may use free or immobilised cells and may operate in batch, fed-batch, repeated fed-batch, or continuous mode and the two components may be linked continuously or discontinuously in an open (exit flow to waste) or recycling system.

In order to reach some conclusions about the efficacy of different strategies, a common comparator is needed. Rates of H_2 production are not always meaningful in this kind of comparison due to number of contributory factors and variables. A common parameter taking into account many factors can be useful, (e.g. H_2 volume produced / reactor volume / dry cell weight / mol substrate consumed / time) but the accurate interpretation of so many factors from published accounts is rarely possible. The molar yield of H_2 from hexose (or monosaccharide) is the most appropriate measure for the comparison of dual systems, as it can be applied regardless of organisms, scale, means of integration and the chemical natures of substrates (Table 1). This factor can be misleading, however, in the case of complex feeds (e.g. algal biomass, tofu wastewater) containing organic acids or non-hexose substrates such as fats and proteins from the outset, which contribute to the fermentative yield of organic intermediates (Ike et al. 1997; Ike et al. 2001).

Additionally, the strategies under evaluation involve an input of solar energy, implying a positive relationship between the system's production capacity and its associated area for absorbing solar energy. Due to the cost and technical challenge of constructing expansive photobioreactors of gas-tight, transparent materials, the energy yield per area of reactor footprint is considered as a second key parameter for comparison.

3.3 Selection of organisms for the 2nd stage

In a dual H_2 producing system, the 2nd stage functions to clean up the effluent from the 1st stage (i.e. to decrease its BOD) and to produce a secondary H_2 stream at the expense of stage 1 products (e.g. reduced organic molecules). An algal-PNS bacterial symbiosis was proposed but no experimental data is yet available (Melis & Melnicki 2006). Fermentation products (e.g. acetate) could be used as C-source for the growth of microalgae, cyanobacteria or PNS bacteria. While acetate is regularly used in algal growth media (Kim et al. 2006c), the authors are not aware of any published attempts to cultivate algae or cyanobacteria using fermentation products as carbon sources. PNS bacteria, conversely, have been cultivated to produce biomass, single-cell protein, or PHB (poly-β-hydroxybutyrate), using primary fermentation waste streams (Ensign 1977; Hassan et al. 1997). Reduced organic molecules are the preferred carbon source for PNS bacteria (Biebl & Pfennig 1981), suggesting that these species may be ecologically associated with fermentative, organic acid-producing organisms. There are many examples of the use of PNS bacteria for H_2 production from wastes (e.g. Thangaraj & Kulandaivelu 1994), many of which have similar characteristics to fermentation effluents.

Using PNS bacteria in the 2nd stage, organic fermentation products can be converted to H_2 with high efficiency (50-100 % of stoichiometric yield) (Rocha et al. 2001) and light conversion efficiencies could approach a hypothetical maximum of 10 % (Akkerman et al. 2002). Using both dairy and sugarcane wastewaters the PNS bacterium *Rhodopseudomonas capsulata* produced H_2 at a 10-fold higher rate than the cyanobacterium *Anacystis nidulans* (Thangaraj & Kulandaivelu 1994).

PNS bacteria are the popular choice for the conversion of organic fermentation products to H_2 in the 2nd stage of a dual system. Of the 37 reports summarised in Table 1, only one employed a purple sulfur bacterium for this purpose (Akano et al. 1996; Ikuta et al. 1997) and none employed microalgae or cyanobacteria, which employ a more energetically demanding mechanism of H_2 production than do APB (Claassen et al. 1999).

3.4: Selection of organisms for the 1st stage

In this overview, dual systems are grouped broadly according to whether both 1st and 2nd stages or only the 2nd stage are light-driven.

3.4.1: Dual systems with photoautotrophic 1st stage

In this approach, photoautotrophs produce H_2 and accumulate carbohydrate during an initial light phase. The photoautotroph switches to fermentative metabolism during a subsequent dark phase, converting starch or glycogen to organic fermentation products, which are utilised by PNS bacteria to generate H_2 in the next light phase. Alternatively, the phototroph cell mass may be harvested to supply the feed for a dual system with dark fermentative 1st stage (section 3.4.2).

1st and 2nd stages may be joined in co-culture or sequentially with transfer of spent broth between stages. Co-culture was until recently unsuitable for this combination because the O_2 produced by microalgae/cyanobacteria would prevent photoproduction of H_2 by PNS bacteria. Microalgal strains exhibiting a decreased rate of photolysis relative to respiration (i.e. decreased rate of O_2 production) have recently become available and work is ongoing to characterise H_2 production in co-cultures of attenuated microalgae and PNS bacteria (Melis & Happe 2004).

In this type of system, there is the possibility for H_2 production in 3 stages because microalgae/cyanobacteria can produce H_2 both at night and by day. While the production of H_2 through photolysis (or nitrogenase) is widely reported (above), there are no accounts of 3-stage systems in which H_2

production occurred in all 3 stages. In all cases, the accumulated biomass was the sole substrate from which H_2 was generated, by dark algal/cyanobacterial fermentation followed by photofermentation.

In most cases, algal fermentation produced no H_2. In the most successful example (Miura et al. 1992), the yield of microalgal fermentation from accumulated starch was *ca.* 1.3 mol H_2/mol hexose and the overall yield was maintained at a steady 10.5 mol H_2/mol hexose for 5 days under continuous illumination (8 mol/mol under diurnal illumination). *Chlamydomonas* spp. were found to accumulate higher levels of starch than other microalgae (Ike et al. 2001) and strain MGA161 with a high fermentative H_2 yield was highlighted (Miura et al. 1986). Although the microalgal-based dual system achieved excellent H_2 yields based on the accumulated carbohydrate, the rate of carbohydrate accumulation limits the application of this strategy as discussed below.

3.4.1.1: Energy generation potential with a photoautotrophic 1st stage

In the case of a dark-fermentative 1st stage fed by wastes or synthetic solutions, the feeding rate can easily be adjusted to control the overall rate of H_2 production, whereas an algal/cyanobacterial-driven system (dependent on light) is limited by the yield of photoautotrophic carbohydrate production (e.g. mol hexose/m^2 light capture area/day). Combined with the molar yield of H_2 *per* hexose in the dual system, this can indicate the potential rate of H_2 production from a given light capture area (Table 3). Using an 800 L pond-type pilot plant with CO_2 as the carbon source for cultivation of *Chlamydomonas* spp., Ikuta et al. (1997) achieved a maximum productivity of 92.6 mmol hexose/m^2/d and an average productivity of 24.4 mmol hexose/m^2/d over 23 days. Using a closed photobioreactor, *Chlamydomonas reinhardtii* was grown under outdoor light conditions using CO_2 in addition to acetate as carbon sources, yielding 158 mmol starch-hexose/m^2/d (Kim et al. 2006c). Assuming cultivation conditions can be optimised to maintain the highest rate of starch accumulation this value was used to calculate the potential productivities of algal/cyanobacterial-driven dual systems (Table 3).

Using the data provided by Levin et al. (2004a) (see legend to Table 3), it can be calculated that at least 436 m^2 of light capture area would be needed to generate sufficient H_2 to power one home with modest energy requirements (1 kW), discounting the energy costs of the process (e.g. mixing, pumping, medium supplements, pH control and maintenance). Thus, significant improvements in the rate and efficiency of light conversion to carbohydrate would be required to permit biological energy generation by a dual system reliant upon microalgal or cyanobacterial starch accumulation, which is rather overshadowed by the availability of significant quantities of starch and cellulose wastes (Yokoi et al. 2002; Haq & Easterly 2006).

As a best-case scenario, metabolic engineering (see above) would lead to significant improvements in the efficacy of direct photolysis, allowing significant H_2 production coupled to carbohydrate accumulation. For decentralised domestic energy generation the available light capture area is estimated to be of the order of 20 m^2. This is challenging since it would require a 20-fold increase in productivity (along with parallel developments in process automation and assuming negligible operational energy costs). Large centralised hydrogen farms might be more efficiently run, but such an industry would be in direct competition with conventional agriculture, which currently accounts for 77 % of land in the UK (Anon 2005). However, H_2 farms would not require fertile soil and might be operated in inhospitable environments such as deserts or on contaminated land where remediation might not be economically attractive (Aldhous 2006). The cultivation of 'energy crops' is currently receiving widespread attention (e.g. de Vrije & Claassen 2003; Aldhous 2006; Schnoor 2006). For example, *Jatropha* spp. are proposed as energy crops suitable for cultivation on sparse, non-arable land for the production of seed oil, which can be esterified to produce bio-diesel fuel with the co-production of 'press-cake' residues which are suitable substrates for bioconversion, e.g. to H_2 (Staubmann et al. 1997; Gübitz et al. 1999; Martínez-Herrera et al. 2006; Tiwari et al. 2007). A comparison of the potential energy yields *per* hectare for crop farms and photo-energy farms would repay study.

As the planet's most plentiful energy source, solar energy must be part of any vision of future energy generation. This is the case either in a wholly light-driven system (e.g. microalgae + PNS bacteria) or in a

partially light driven system (fermentation + PNS bacteria) where the fermentation is fed on biomass residues. As world population and food-demand grow, the limited availability of non-agricultural land may discourage algal or cyanobacterial cultivation. While 'green roofs' are established as a means of improved insulation and storm water retention, the potential of rooftop agriculture remains to be widely exploited (Nowak 2004). At the same time, the availability of residues is set to increase from both food and energy crops. Therefore, with the current state of knowledge and technology development, a dual system with dark fermentative 1st stage has a greater potential for near-term application.

On a broader scale, the use of microbial photosynthesis will have to compete with photovoltaic (PV) technology which, although as yet economically unattractive in most applications, is also under parallel development (Avi 2007). Data are, as yet, unavailable to compare the energy yields from optimised PV and bio-systems as industrial-scale photobioreactors for H_2 production are still under development. The estimated net energy ratio (NER) was *ca.* 2 for a photobioreactor lasting 20 years, constructed using tubes of flexible polyethylene film (thickness 0.18 mm), under the assumptions of film replacement every 3 years, 80 % time on-line, 20 % loss of H_2 and discounting the costs of nutrients, temperature control and water (Burgess & Fernandez-Velasco 2007).

3.4.2: Dual systems with a dark fermentative 1st stage

Dark fermentation represents a rapid and relatively simple method for the conversion of carbohydrates into hydrogen, but the accumulation of organic fermentation products can exert stress upon the fermenting microorganisms and generates a secondary waste, requiring disposal (Eiteman & Altman 2006). Concurrently, fermentation products are preferred substrates for PNS bacteria, which oxidise reduced organic molecules and dispose of the reducing power as H_2. It has long been recognised that dark fermentation and photo-fermentation should be coupled to create an efficient scheme for waste-free hydrogen production (Odom & Wall 1983; Miyake et al. 1984). In practice, the maximum yield reported was 8.3 mol H_2/mol hexose (Kim et al. 2006c) and indeed several independent results of *ca*. 7 mol H_2/mol hexose were generated by different methods (Table 1).

The dark fermentation-photofermentation (DF-PF) dual system can be operated in continuous mode over extended periods. The longest experiment reported sustained H_2 evolution for 45 days by coupling lactic acid fermentation and a continuous photofermentation, but the yield of H_2/mol hexose cannot be calculated from the available data (Franchi et al. 2004). Yokoi et al. (2001 and 2002) reported sustainable operation of a dual system for 30 days, maintaining a steady overall yield of 7 mol H_2/mol hexose, using sweet potato starch residue. This system was used in repeated-batch culture, the fermenter being partially drained daily and the photobioreactor every 5 days. A fully continuous system is currently under development (Redwood & Macaskie 2007a, 2007b). A continuous *E. coli* CSTR and a continuous *R. sphaeroides* photobioreactor were integrated by anion-selective electrodialysis, simultaneously transferring anionic fermentation products, while retaining repressive ammonium ion, *E. coli* cells and suspended solids. This approach resulted in sustained H_2 production by *E. coli* with a yield of 1.6 mol H_2/mol hexose and sustained H_2 photoproduction by *R. sphaeroides* despite the presence of 15 mM ammonium ion in the initial feed. The overall yield was 2.4 mol H_2/mol glucose, attributable to a low efficiency in the PBR (38 %) and a proportion of *E. coli* products being uncharged species (ethanol), not transported by electrodialysis. An overall yield of 10.1 mol H_2/mol glucose could be predicted based on a substrate conversion efficiency of 75 % in the photobioreactor and optimisation of the latter is in progress.

Therefore, with present approaches, a dual system can be sustained continuously and achieves on average *ca*. 60 % of the hypothetical maximum, 12 mol H_2/mol hexose (Figure 2D). As a priority, research is needed to investigate techniques for the integration and rate-balancing of inter-dependent bioreactors, alongside further study of the conditions needed to sustain high H_2 production continuously in either component of DF-PF dual systems.

3.4.2.1 Selection of organism for dual systems with dark fermentative 1st stage

An ideal fermentation, coupled to an ideal photo-fermentation could approach the maximum stoichiometry of 12 mol H_2/mol hexose (Figure 2D). As different fermentations can theoretically be coupled to a photofermentation to achieve the same maximum yield (Figure 2), differences in practicality and experimental yields must be addressed.

The distinction is made between the use of obligate anaerobes in stage 1 and facultative aerobes/anaerobes because there are distinct differences between the biochemical mechanisms of H_2 production of these classes (section 2.2, Figure 2).

In the case described by Yokoi et al. (2001 and 2002), the facultative anaerobe *Enterobacter aerogenes* was included in the 1st stage in co-fermentation with the strict anaerobe *C. butyricum* (Table 1). This example is classed among the strict anaerobic dual systems because *Enterobacter*, being unable to utilise starch, did not contribute to the fermentation but provided a cheaper alternative to reducing agents to ensure anaerobiosis by scavenging O_2 (Yokoi et al. 2001; Yokoi et al. 2002).

Facultative anaerobes (e.g. *E. coli*) can be pre-cultured rapidly, are readily amenable to metabolic engineering and do not require the addition of a reducing agent to 'poise' the redox potential, while the biochemistry of mixed-acid fermentation has been well-studied (Stephenson & Stickland 1932; Knappe 1987; Alam & Clark 1989; Clark 1989; Bock & Sawers 1992; Vardar-Schara et al. 2008). Nevertheless, obligate anaerobes have been preferred in the study of dual systems, perhaps due to the potentially higher H_2 yield. Table 1 shows 14 examples of strict anaerobe-driven dual systems, with an average overall yield (where given) of 5.73 mol H_2/mol hexose (or 47.8 %). Conversely only eight examples of dual systems could be found using other types of fermentation in the 1st stage, of which systems based on lactic acid fermentation were the most effective, producing (overall) up to 7.3 mol H_2/mol hexose entirely from the 2nd stage (Kawaguchi et al. 2001). High overall yield is, therefore, possible without H_2 production in the 1st stage. This can be explained by the fact that lactic acid fermentation has been optimised for the industrial production of lactic acid (Li et al. 2004) and because lactate is theoretically converted to 6 H_2 in the 2nd stage, which typically operates with high efficiency (section 2.1.2).

It is possible that researchers have frequently disregarded mixed-acid fermentation for use in the 1st stage because of its theoretically lower H_2 yield (Figure 2). The fermentative yield of H_2 from hexose by living organisms is thermodynamically limited to 4 mol H_2/mol hexose (Thauer 1977). The metabolic pathways of strict anaerobes (e.g. *C. butyricum*) allow this to be achieved only under very low H_2 partial pressure, otherwise the reaction is stoichiometrically similar to mixed-acid fermentation, producing a maximum of 2 mol H_2/mol hexose (Figure 2). The yield from strict anaerobic fermentation in a dual system has not exceeded 2.6 mol/mol (Table 1) and the use of a non-sporulating facultative anaerobe would represent an insignificant sacrifice of yield for a significant reward in practicality (Figure 2D, section 3.4.2.1).

Hyperthermophilic fermentations may yield up to 3.8 mol H_2/mol hexose in practice (section 2.2.1), but no accounts describe dual systems using hyperthermophiles. Furthermore, they would not be compatible with PNS bacteria in co-culture, which produce H_2 optimally at *ca.* 30 ºC and live at temperatures below 47 ºC (Castenholz 1995). A hypothetical industrial-scale facility based on the sequential combination of a thermobioreactor and a photobioreactor was estimated to produce H_2 at a cost of €2.74 /kg H_2 (de Vrije & Claassen 2003). If the bio-H_2 were used to generate electricity in a fuel cell operating at 50 % efficiency and 95 % utilisation, the cost of the energy production would be €0.145 /kWh. The price of domestic electricity is *ca.* €0.25-0.30 /kWh (2008, www.britishgas.co.uk). However a more extensive economic analysis of the bioprocess, suggested a cost of €4/kg H_2 (Davila-Vazquez et al. 2008a). Continued research and development of bio-hydrogen systems is required preceding a reliable economic assessment.

Some information is available regarding the use of non-axenic fermentations in the 1st stage of a dual system (Table 1). This strategy takes advantage of the presence of suitable microorganisms in the feedstock, thus eliminating the need to sterilise inputs and to pre-culture specific organisms. However, in undefined mixtures of microorganisms it is more difficult to repress unwanted reactions (section 2.2.2), H_2 yields are

generally lower than in axenic dual systems (Table 1) and it would be difficult to ensure reproducibility between feedstock sources and locations.

3.4.2.2: Distribution of H_2 production among stages of a dual system

Ike et al. (1997) compared 3 different methods of H_2 generation from algal biomass rich in starch, of which the most effective (in terms of H_2 yield) involved a lactic acid fermentation (producing no H_2) followed by photo-fermentation with PNS bacteria, showing that it is not essential to produce H_2 in both phases of the dual system.

Figure 2 illustrates that various different stage 1 fermentations (e.g. lactic acid, mixed-acid and clostridial-type fermentations) can be applied with different H_2 yields but with equal potential for H_2 production overall (12 mol H_2/mol hexose). The type of fermentation employed affects the theoretical distribution of H_2 between the 1st and 2nd stages. If lactic acid fermentation were used, all 12 moles of H_2 would arise from the 2nd stage; 10 for mixed-acid fermentation and 8-10 for *C. butyricum*.

It is possible that *C. butyricum*-based dual systems have been favoured by researchers in order to skew the distribution of H_2 production towards the 1st stage and thus to minimise the required transfer of organic fermentation products and the light capture area. Conversely, H_2 is generally produced by fermentations at *ca.* 50 % of the theoretical maximum, while the photo-fermentation typically operates at *ca.* 70 % efficiency, so a higher overall yield could be expected using lactic acid or mixed-acid fermentation in which more H_2 is produced in the more efficient 2nd stage. The gain in productivity should be offset against the increased costs of light capture area and transfer of fermentation products.

3.4.2.3 Energy generation potential with a dark fermentative 1st stage.

The increased productivity of a dual system over a single-stage system is significant. For example, a molasses-fed pilot fermentation plant generated 8240 L H_2 (342.5 mol H_2) and 3000 L effluent *per* day (Ren et al. 2006). The effluent contained primarily acetate and ethanol with a hydrogen production potential of 246.35 mmol H_2/L (authors' calculations). Therefore the addition of a photoheterotrophic 2nd stage could maximally increase productivity by 317 %.

As a best-case scenario, a dual system capable of generating 12 mol H_2/mol hexose might be developed. Work is underway to meet a target of 10 mol H_2/mol hexose, which would make bio-H_2 economically viable, given low feedstock costs (Davila-Vazquez et al. 2008a). Using reported values for the productivity of dual systems, the potential for energy generation can be estimated.

With the same assumptions as used in Table 3, a household might consume H_2 at a minimum rate of 573.6 mol/d (Levin et al. 2004a; Levin 2004b). The feasibility of the decentralised application of a sequential dual system was evaluated (in this study) by calculating the necessary reactor sizes and the feed requirements to meet this demand. The energy requirements of the process were not taken into account.

If the potential H_2 yield (12 mol H_2/mol hexose) were to be distributed 4:8 between the 1st and 2nd stages, respectively, then the dark fermentation would be required to produce 191.2 mol H_2/d and the photobioreactor 382.4 mol H_2/day (of which only *ca.* 12 h is light). Using published volumetric productivities (Levin et al. 2004a; Levin 2004b), it was calculated that a 79.6 L fermenter (containing an undefined mesophilic culture, *ca.* 0.1 mol H_2/L/h) would be needed. The productivity of the photobioreactor (PBR) would be constrained by several parameters: the limited efficiency of light conversion to H_2 (10 % maximum; Akkerman et al. 2002), light availability (1 kW/m^2 for 12 h/d; Miyake et al. 1999), specific rate of H_2 production (ca. 0.1 L H2/g dw/h; Rocha et al. 2001), culture depth (*ca.* 5 cm), culture density (*ca.*1 g dw/L : OD *ca.* 2.5). To operate within these constraints a PBR volume of 7648 L would be required. The corresponding square panel PBR could be 12.4 m wide with a light capture area of 153.0 m^2 and a depth of 5

cm; sufficiently shallow to maintain the entire culture under saturating light intensity (Nakada et al. 1995; Katsuda et al. 2000). This area could potentially harvest 6.6 GJ *per* 12 h light period, indicating a comfortable light conversion efficiency of 1.65 % to meet the H_2 demand (Figure 4).

It is noteworthy that the limiting factor is the specific rate of H_2 production (necessitating a dense culture and limiting the PBR depth) rather than the light conversion efficiency even at the reasonable light intensity of 1 kW/m^2. Were specific rates to be increased (e.g. through improved strains or bioreactors) the light intensity and conversion efficiency would then limit the productivity of the photobioreactor. At a light intensity of 1 kW/m^2 for 12 h *per* day, with 65.8 % of useful solar energy and a light conversion efficiency of 10 % the corresponding light capture area to produce 382.4 mol H_2 in 12 h would be of 38.44 m^2 (6.2 m x 6.2 m). A house might barely accommodate an 80 L fermenter and a 40 m^2 photobioreactor, along with H_2 storage, regulatory equipment and fuel cell (Figure 4), but even with a conversion efficiency of 12 mol H_2/mol hexose, the feed-demand would be 7.74 kg cellulose/d or 8.17 kg sucrose/d, which could be supplemented with organic household wastes for disposal, although the additional sugar from this source would be negligible. Sugar production is an agricultural industry, so this option could not be applied in the long-term due to competition for farmland (as 3.4.1.1) but wastes from sugar production and processing could be exploited as feed substrates.

Whereas the cultivation of energy-dedicated crops to generate sugars for conversion to H_2 would incur costs, it could be economically realistic to co-locate H_2 production with feed sources such as food processing plants. The UK food industry produces *ca.* 5.3 million tonnes of biodegradable waste annually, a large fraction of which is disposed of by land-filling, incurring both economic and environmental costs (Anon 2004; Bartelings et al. 2005). Co-locating food-waste generation and conversion to H_2 would remove transport and disposal costs, while minimising spoilage of the residues to maintain their value. Bio-H_2 production could be optimised for the use of residues having relatively consistent composition and little H_2 storage or distribution would be required as produced energy could be used on-site to meet predictable energy demands and any excess production could be sold to alleviate the demand for fossil fuel.

There are many accounts of bio-hydrogen production from non-synthetic substrates (i.e. wastes) and dual systems have been applied in several cases (Table 1) (Zhu et al. 1995; Fascetti et al. 1998; Kim et al. 2001; Zhu et al. 2002; Franchi et al. 2004). De Vrije and Claassen (2003) described a hypothetical process fed by lignocellulosic biomass and calculated that 9 % of the domestic energy demand could be met using available biomass residues in the Netherlands. However, the authors are not aware of any currently operating economically viable bioprocess based on lignocellulosic feedstocks.

3.4.2.4 Bio-H_2, bio-methane or bio-ethanol?

Biomass residues are available in significant quantities for use as feedstocks for bioenergy production (Easterly & Burnham 1996; Filho & Badr 2004; Haq & Easterly 2006; Mabee et al. 2006; Dawson & Boopathy 2007; Levin et al. 2007). Bioprocesses for the production of H_2, methane and ethanol can all utilise biomass residues as feeds, although currently, bio-ethanol and bio-methane processes are commercially more advanced than bio-H_2 processes.

Levin et al. (2007) calculated the energy potential of Canada's biomass residues for methane production by anaerobic digestion and H_2 production by anaerobic bacterial fermentation. The potential H_2 energy equated to only 41.4 % of the potential methane energy. However, this calculation was based on a H_2 yield of 1.3 mol H_2/mol hexose from a single-stage bacterial fermentation. Several authors report multi-organism systems for H_2 production producing in excess of 7 mol H_2/mol hexose (Weetall et al. 1989; Miura et al. 1992; Ike et al. 2001; Kawaguchi et al. 2001; Yokoi et al. 2001; Asada et al. 2006; Kim et al. 2006c). Hence, a bio-H_2 process could be more energetically productive than a bio-methane process if dual H_2-producing systems could be implemented. With H_2 as the final product, bio-methane could be converted by steam reforming, which is a well-established thermocatalytic process. However, this would introduce significant parasitic energy demand due to the required high temperature (*ca.* 700 °C). Furthermore reformed H_2 is contaminated with CO (a potent catalyst poison) and would require extensive purification in

order to be made comparable in quality to Bio-H_2. Therefore, a dual H_2-producing system would offer the comparative advantages of significantly greater energetic productivity and applicability to fuel cells.

Bio-ethanol is a major energy vector in Brazil, with a production of 16 billion L of ethanol annually, requiring *ca.* 3 million hectares of land. The total sugarcane crop area (for sugar and ethanol) is 5.6 million hectares (Goldemberg 2007). The average industrial yield from the crop of 2004/2005 was 144.35 kg sucrose/tonne sugarcane, equivalent to 79.39 L of anhydrous ethanol/tonne sugarcane or 82.86 L hydrous ethanol/tonne sugarcane (Nastari et al. 2005). Therefore, the process efficiency of bio-ethanol production is 80.5 % (of a biochemical maximum of 2 mol ethanol/mol hexose). Considering the higher heating value (HHV) of ethanol of 29.840 MJ/kg (http://hydrogen.pnl.gov/cocoon/morf/hydrogen/article/401), the bio-ethanol process produces 2,212 kJ energy/mol hexose (from sugar cane). To equal this energy yield a bio-hydrogen process must achieve *ca.* 7.8 mol H_2/mol hexose (HHV of H_2 = 141.88 MJ/kg, hydrogen.pnl.gov/cocoon/morf/hydrogen/article/ 401). This could not be achieved by a single-organism system and a dual system would be required.

4: Conclusions and future perspectives

Biological hydrogen production is a promising avenue that should be pursued urgently as the world energy demand increases, fossil fuel resources dwindle and the need for greenhouse gas minimisation becomes increasingly pressing. Hydrogen biotechnology is poised to become increasingly prominent alongside, and eventually emerging as competitive with other sustainable bio-fuel processes and/or as an adjunct to them.

This review shows that (unlike with bio-ethanol production) no single microorganism can produce competitive yields of H_2. Multiple-organism systems offer increased H_2 yields and would be mandatory for realistic future energy generation.

Examination of the properties of photosynthetic microorganisms revealed that purple non-sulfur (PNS) bacteria are the most suitable organisms for the 2nd stage of a dual system, while for the 1st stage dark fermentation, clostridia have been the most widely used, but facultative aerobes may increase the ease of operation while detracting little from the overall H_2 yield.

A dual system combining anaerobic fermentation and photoheterotrophy could potentially result in high energy yields from industrial wastes or biomass residues, although it is unlikely that a domestic household would produce sufficient fermentable waste to make a significant contribution to its energy budget. For example, the power output of a pilot fermentation plant neared the demand of 1 household, processing daily *ca.* 4.4 tonne of diluted feedstock (molasses, 3 g COD/L) to generate 343 mol H_2 (Ren et al. 2006), sufficient to produce a constant electrical power output of 0.6 kW using a realistic PEM-FC (operating at 50 % efficiency and 95 % utilisation; Levin et al. 2004a).

Even by increasing the output by several-fold by addition of the second stage PBR it is unlikely that a light-driven dual system would repay investment for single household domestic electricity generation. Furthermore, it can be calculated that for domestic self-sufficiency several tonnes of sugary waste would be required annually, therefore, substrate supply would be the limiting factor rather than spatial considerations. Hence, industrial, retail and agricultural waste producers would be the likely initial users of bio-H_2 systems.

In addition to food processing and retailing wastes, biomass residues are available in significant quantities for use as feedstocks for bioenergy production (Easterly & Burnham 1996; Filho & Badr 2004; Haq & Easterly 2006; Mabee et al. 2006; Levin et al. 2007). Bioprocesses for the production of H_2, methane and ethanol can all utilise biomass residues as feedstocks although, currently, bio-ethanol and bio-methane processes are commercially more advanced than bio-H_2 processes.

It was argued above (section 3.4.2.4) that a dual bio-H_2 system could be more productive than bio-methane and equally productive to bio-ethanol in terms of energy production, but these calculations were based on two noteworthy assumptions. Firstly, this calculation did not take into account parasitic energy losses, such as the distillation cost incurred by ethanol recovery (*ca.* 25-33 % of the product's combustion

value; Kvaalen et al. 2006) or the operating costs of relatively complex dual systems for H_2 production, which cannot be estimated due to the immaturity of this technology. Secondly the assumption was made that energy can be recovered from H_2 and CH_4 with equal efficiency, e.g. by coupling of the bio-gas-producing generation reactor to a fuel cell for electricity generation assuming a pure gas stream (e.g. see Macaskie et al. 2005). The most efficient type, proton exchange membrane (PEM; also called polymer electrolyte membrane) fuel cells, achieve the highest power densities when H_2 is used as a fuel, whereas solid oxide fuel cells (SOFCs) are more suitable for the use of hydrocarbons such as methane (Larminie & Dicks 2003). However SOFCs use an oxide ion-conducting ceramic material as the electrolyte and require an operating temperature of 600-1100 °C and hence the necessary heat input detracts from the overall energy balance. In addition, any contamination of bio-methane with H_2S, the end product of dissimilatory sulfate metabolism by the sulfate-reducing bacteria present in anaerobic mixed cultures, would necessitate gas filtration, since sulfur compounds are powerful catalyst poisons affecting all types of fuel cells. The direct methanol fuel cell (DMFC) is a type of PEM-FC in which methanol reacts (slowly) at the anode according to: $CH_3OH + H_2O \rightarrow 6H^+ + 6e^- + CO_2$ (Larminie & Dicks 2003). For DMFC, the power density is relatively low compared to PEM-FC and SOFC, but this would not prevent application in portable devices such as laptop computers, where the power storage exceeds 0.025 kWh and the required DMFC unit would be significantly smaller in volume than the equivalent lithium-ion battery (see Larminie & Dicks, 2003).

The formation of methanol from methane *via* methane monooxygenase is very well established (Grosse et al. 1999; Dalton 2005) and a comparative study of the various bio-gas and fuel cell-coupling options would be worthwhile.

Fuels which are liquid at ambient temperature (e.g. methanol and ethanol) have higher volumetric energy densities than gaseous H_2. Apart from the consideration of land use the long-term economics of bio-ethanol production should be considered (Rogers et al. 2005). Ethanol cannot be used efficiently in fuel cells (Larminie & Dicks 2003) and a significant problem is considered to be the higher cost of bio-ethanol production (from cellulosic biomass) as compared to diesel or petrol. The price of bio-ethanol was projected to become comparable to that of petrol by 2015, based on a conservative forecasted price of $35-$40 *per* barrel crude oil (Chandel et al. 2007), but this may occur sooner due to spiralling oil prices ($135 *per* barrel in May 2008 and climbing; www.bloomberg.com). The distillation cost of ethanol is significantly higher at low ethanol concentrations (Zacchi & Axelsson 1989) and a membrane distillation process can be used as an efficient and cost effective option (see Chandel et al. 2007); molecular sieve techniques are now widely used in the industry (Rogers et al. 2005). A net energy balance (NEB) calculated by Hill et al. (2006) suggested that corn grain ethanol provides *ca.* 25 % more energy than that consumed in its production; however, almost all of the NEB can be attributed to the 'energy credit' for the animal feed co-product.

Such calculations are moderated according to the geographic region. Hence, Brazil has certain comparative advantages in ethanol production. Unlike American or European processes based on crops (e.g. barley, corn or wheat) that must first be converted at significant expense into fermentable sugars, Brazilian (and also Australian) processes are based on sugarcane which the climate favours, obviating any need for conversion. Ethanol produced from sugarcane in Brazil has a net positive energy balance (renewable energy output *versus* fossil fuel input) of 10.2, whilst the equivalent value for ethanol from corn (US) is 1.4 (Goldemberg 2007). Also, the production cost of ethanol from sugarcane (Brazil) ($0.81 *per* gallon, in 2006) is lower compared to ethanol from corn (US) ($1.03 *per* gallon, in 2006) and is competitive with gasoline in the US, even considering the import duty of $0.54 *per* gallon and energy-efficiency penalties (30 % or less with modern flexible fuel vehicle technologies) (Goldemberg 2007). Ethanol produced in Brazil has remained fully competitive with gasoline on the international markets, without government intervention, since 2004, i.e. subsidising ethanol production is a thing of the past. In addition to the production of ethanol, the industrial processing of sugarcane generates bagasse, a valuable product which adds to the industry's positive environmental differential because it has been used to replace fossil fuels in the production of industrial heat and electricity in the sugar mills and distilleries, thereby boosting the abatement potential of greenhouse gases emission (Macedo et al. 2004). Moreover, the competition for land use between food and fuel has not been substantial: sugarcane covers 10 % of total cultivated land but only 1 % of total land available for agriculture in Brazil (Goldemberg 2007).

A recent review (Hill et al. 2006) has evaluated critically the long-term potential for bio-ethanol against emerging bio-diesel technology. While ethanol is made by the fermentation of biomass substrates (cane sugar is ideal because no further processing is required), bio-diesel is made *via* processing of plant material from 'energy crops'. For example, soybean bio-diesel is sourced directly from long-chain triglycerides obtained from the seeds; in comparison corn-starch requires pre-enzymatic conversion into fermentable sugars for ethanol production. Critically, bio-diesel yields 93 % more energy than that invested in its production and, relative to the fossil fuels they displace, greenhouse gas emissions are reduced by 12 % and by 41 % by bio-ethanol and bio-diesel, respectively. However, Hill et al. (2006) point out that 'even dedicating all U.S. corn and soybean production to bio-fuels would meet only 12 % of gasoline demand and 6 % of diesel demand'. Lin & Tanaka (2006) suggest that any country with a significant agronomic-based economy could use technology for ethanol fuel production. However, this and many other critiques overlook the difficulty of achieving a positive energy balance for the production of bio-ethanol from crops such as corn and wheat rather than from sugarcane. Whereas the result of recent studies was a positive energy balance of 1.4 (Goldemberg 2007), the cultivation area needed to support a US fuel economy based on corn-ethanol would equate to most of the nation's land area (Pimentel 2001) and the same argument applies to all 'energy crops' that compete for agricultural land with food supply, a very major factor, which is acknowledged but understated by Hill et al. (2006). These authors suggest the use of agriculturally marginal land or the use of waste biomass for bio-ethanol production; both are potentially more sustainable than outright energy crop cultivation. Assuming that the cost of ethanol recovery can be lowered by effective recovery technology, the use of large global reserves of lignocellulosic waste biomass as potentially fermentable feedstock is receiving widespread attention with respect to bio-ethanol production and also with respect to bio-H_2 production (de Vrije & Claassen 2003; Aldhous 2006; Schnoor 2006). The main problem lies in converting the recalcitrant woody material into readily fermentable substrate. This requires pre-treatment, which may be physico-chemical, enzymatic or combinations of these. An overview of upstream treatments is outside the scope of this review and the reader is referred to recent example reviews in this area (Rogers et al. 2005; Lin & Tanaka 2006; Chandel et al. 2007). Once a fermentable feedstock is generated there are several options for the downstream energy production process and the hydrolysate could be equally well used for bio-hydrogen production as for bio-ethanol production, without the attendant processing costs.

The use of energy crops for bio-diesel production is particularly promising and the technological limitations have been reviewed by Abdullah et al. (2007). Chemically, 'bio-diesel' is fatty acid methyl esters, produced by the transesterification of oils and fats with methanol in the presence of suitable catalysts. Here bio-methanol could find a large-scale application as an alternative to the niche market for fuel cell use. The disadvantages of bio-diesel production are that large volumes of contaminated wastewater are produced and that homogeneous catalysis is employed for maximum processing efficiency; the catalyst is currently not retained and major research efforts are directed towards the development of solid phase catalysts (Abdullah et al. 2007). Glycerol is produced in tonnage quantities as a by-product, which could be a suitable substrate for microbial fermentation to produce ethanol or H_2 as additional energy products. However, the glycerol is obtained as an aqueous impure NaCl-solution which requires purification and its use as a fermentation substrate would compete with other potential uses in the pharmaceutical, cosmetic and food industries and as animal feeds, polymers, surfactants and lubricants (Ma & Hanna 1999). Assuming that microorganisms resistant to the contaminants are developed, bio-hydrogen production could be attractive in this context since a gaseous product is easily separated from the fermentation liquor and hence the purity or otherwise of the starting material is largely irrelevant, assuming the product gas stream is free of volatile agents.

Bio-diesel is made from waste oils or by pressing plant material (e.g. seeds) to extract the oils and hence plant residua could be a useful waste for fermentation to make a secondary ethanol or hydrogen fuel stream; however, the problems of upstream treatment of the wastes are similar to those of other fibrous materials (above), although in at least one example waste from oil production (in this case olive oil) has been used as the substrate for bio-hydrogen production (Eroğlu et al. 2004). Clearly the use of edible oils from food-crops such as *Olea* spp. (olive) for bio-fuel production would be impractical, however attention has recently focused on the use of inedible oil for bio-diesel production, obtained from the tropical oil seed

plant *Jatropha curcas*, which is drought-resistant and can grow on marginal, sub-arable or even waste land (Srivastava & Prasad 2000) in Central and South America, Mexico, South-East Asia, India and Africa. *J. curcas* is not suitable as animal feed without detoxification (Martínez-Herrera et al. 2006) but has many other applications and a transesterification process of the seed oil as a bio-fuel has been evaluated on an industrial scale (1500 tonnes per annum; Gübitz et al. 1999). Due to its high free fatty acid content (*ca.* 14 % w/w) *Jatropha* oil requires pre-esterification using methanol before conventional transesterification to produce bio-diesel, which was shown to give a high yield of bio-diesel with satisfactory fuel properties (Tiwari et al. 2007). Bio-methane production from anaerobic digestion is a potential source of bio-methanol, which could find use in the pre-esterification reaction. Crushing *Jatropha* seeds to release the oil results in an equal mass of press-cake, which can be used as a substrate for further bioprocessing, e.g. methane production by anaerobic digestion (Staubmann et al. 1997) or, indeed bio-H_2 production although this has not been attempted to date.

In conclusion, the production of bio-fuels (bio-diesel, bio-ethanol or bio-gases) from energy-dedicated crops appears to be unsustainable unless the plant occupies a niche other than agricultural land or provides a high yield of energy *per* area of cultivation. Agricultural residues (lignocellulosic biomass) are available as sources of fermentable substrate for bio-fuel production but the conversion of these wastes into fermentable substrate forms a common bottleneck. Bio-gases and bio-ethanol can both be made by the fermentation of sugars and sugary wastes but the processing costs of ethanol limit the energy output of this method.

The hydrogen economy *per se* is still some decades away but combination and hybrid technologies are appealing in the shorter term. Production of H_2 from food waste sources or from the wastes from bio-diesel production is potentially a clean and sustainable route to clean energy production.

Although the maximum yields of H_2 from sugar are being approached by fermentation this is only possible by the application of more than one microorganism. This review has attempted to identify the two-stage approaches by which maximum yields (and rates) of conversion can be obtained and it identifies that, as with energy crops, available land area for light capture is likely to be a major limiting factor in operation. Under-used, waste ground in sunny regions (as for *Jatropha*) could provide one solution but for most of the developed world arable land takes priority for food production. Process intensification is required to overcome the problem of light delivery to the second stage photofermentations, which would push bio-H_2 production to competitive levels. A review of photobioreactor designs to achieve effective light transfer into high-activity cultures is outside the scope of this overview; the reader is referred to recent reviews (Tsygankov 2001; Hoekema et al. 2002; Kondo et al. 2002; Hoekema et al. 2006; Claassen & de Vrije 2007).

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6: Bibliography

Abdullah AZ, Razali N, Mootabadi H & Salamatinia B (2007). Critical technical areas for future improvements in biodiesel technologies. Environ Res Lett 2:034001 doi:10.1088/1748-9326/2/3/034001.

Akano T, Miura Y, Fukatsu K, Miyasaka H, Ikuta Y, Matsumoto H, Hamasaki A, Shioji N, Mizoguchi T, Yagi K & Maeda I (1996). Hydrogen production by photosynthetic microorganisms. Appl Biochem Biotechnol 57-8:677-688.

Akkerman I, Janssen M, Rocha J & Wijffels RH (2002). Photobiological hydrogen production: photochemical efficiency and bioreactor design. Int J Hydrogen Energy 27(11-12):1195-1208.

Alam KY & Clark DP (1989). Anaerobic fermentation balance of *Escherichia coli* as observed by *in vivo* nuclear magnetic resonance spectroscopy. J Bacteriol 171(11):6213-6217.

Aldhous P (2006). Green gold. New Sci 25 Feb:37-40.

Anon (2004). United Kingdom Food and Drink Processing Mass Balance: Biffaward Programme on Sustainable Resource Use. C-Tech Innovation, Capenhurst, UK.

Anon (2005). Agriculture in the United Kingdom. Retrieved Aug 2007, 2007, from statistics.defra.gov.uk/esg/publications/auk/2005/default.asp.

Antal TK & Lindblad P (2005). Production of H_2 by sulphur-deprived cells of the unicellular cyanobacteria *Gloeocapsa alpicola* and *Synechocystis* sp. PCC 6803 during dark incubation with methane or at various extracellular pH. J Appl Microbiol 98(1):114-20.

Aoyama K, Uemura I, Miyake J & Asada Y (1997). Photosynthetic bacterial hydrogen production with fermentation products of cyanobacterium *Spirulina platensis*. International Conference on Biological Hydrogen Production, Waikoloa, Hawaii, Plenum Press, New York.

Asada Y, Tokumoto M, Aihara Y, Oku M, Ishimi K, Wakayama T, Miyake J, Tomiyama M & Kohno H (2006). Hydrogen production by co-cultures of *Lactobacillus* and a photosynthetic bacterium, *Rhodobacter sphaeroides* RV. Int J Hydrogen Energy 31(11):1509-1513.

Avi S (2007). Photovoltaics literature survey (No. 51). Progress in Photovoltaics: Research and Applications 15(1):87-91.

Bae J-H, Bardiya N & Reddy MRVP (2005). Bio-hydrogen: technology and future prospects. Wealth from waste: trends and technologies. Lal B & Reddy MRVP, (Eds). New Delhi, India, TERI Press**:** 87-132.

Banik RM, Santhiagu A, Kanari B, Sabarinath C & Upadhyay SN (2003). Technological aspects of extractive fermentation using aqueous two-phase systems. World J Microbiol Biotechnol 19(4):337-348.

Bartelings H, van Beukering P, Kuik O, Linderhof V, Oosterhuis F, Brander L & Wagtendonk A (2005). Effectiveness of landfill taxation, Report prepared for the Dutch Ministry of Housing, Spatial Planning and the Environment, Institute for Environmental Studies, Vrije Universiteit, R-05/05, Amsterdam. Available at www.ivm.falw.vu.nl/research_output/index.cfm/home_subsection.cfm/subsectionid/FF91BCBD-EAFE-426A-ABB8184073A39BBF.

Basak N & Das D (2007). The prospect of purple non-sulfur (PNS) photosynthetic bacteria for hydrogen production: the present state of the art. World J Microbiol Biotechnol 23:31-42.

Benemann JR (1996). Hydrogen biotechnology: Progress and prospects. Nat Biotechnol 14:1101-1103.

Bevan A, Book D, Züttel A & Harris R (2007). The protium project: A hybrid electric canal boat using metal hydride store and a PEM fuel cell. The Oxford-Kobe Energy Seminar, 22th-24th April 2007. Hydrogen Storage: The International Grand Challenge, Kobe Institute, Japan.

Biebl H & Pfennig N (1981). Isolation of members of the family *Rhodospirillacae*. The Prokaryotes. Starr MP, Stolp H, Truper HG, Balows A & Schlegel HG, (Eds). Springer. 1**:** 267-273.

Blankenship RE, Madigan MT & Bauer CE (1995). Anoxygenic photosynthetic bacteria. Dordrecht, The Netherlands, Kluwer Academic.

Bock A & Sawers G (1992). Fermentation. *Escherichia coli* and *Salmonella Typherium*. Niedhardt FC, (Ed.). Washington DC, ASM Press. 1**:** 262-282.

Borodin VB, Tsygankov AA, Rao KK & Hall DO (2000). Hydrogen production by *Anabaena variabilis* PK84 under simulated outdoor conditions. Biotechnol Bioeng 69(5):478-485.

Burgess G & Fernandez-Velasco JG (2007). Materials, operational energy inputs, and net energy ratio for photobiological hydrogen production. Int J Hydrogen Energy 32:1255-1234.

Carrieri D, Ananyev G, Garcia Costas AM, Bryant DA & Dismukes GC (2008). Renewable hydrogen production by cyanobacteria: Nickel requirements for optimal hydrogenase activity. Int J Hydrogen Energy 33(8):2014-2022.

Castenholz RW (1995). Ecology of thermophilic anoxygenic phototrophs. Anoxygenic photosynthetic bacteria. Blankenship RE, Madigan MT & Bauer CE, (Eds). Dordrecht, The Netherlands, Kluwer Academic**:** 87-103.

Chandel AK, Chan ES, Rudravaram R, Narusu ML, Rao LV & Ravindra P (2007). Economics and environmental impact of bioethanol production technologies: an appraisal. Biotechnol Mol Biol Rev 2(1):14-32.

Chen X, Sun Y, Xiu Z, Li X & Zhang D (2006). Stoichiometric analysis of biological hydrogen production by fermentative bacteria. Int J Hydrogen Energy 31:539-549.

Claassen PAM, van Lier JB, Lopez-Contreras AM, van Niel EWJ, Sijtsma L, Stams AJM, de Vries SS & Weusthuis RA (1999). Utilisation of biomass for for the supply of energy carriers. Appl Microbiol Biotechnol 52:741-755.

Claassen PAM, Budde MAW, Van Noorden GE, Hoekema S, Hazewinkel JHO, Van Groenestijn JW & De Vrije GJ (2004). Biological hydrogen production from agro-food by-products. Total Food: Exploiting co-products - minimizing waste, Institute of Food Research, Norwich, UK.

Claassen PAM & de Vrije GJ (2007). Hydrogen from biomass. Public report, BWP II project, Agrotechnology and Food Sciences Group, Wageningen. 2007.

Clark DP (1989). The fermentation pathways of *Escherichia coli*. FEMS Microbiol Rev 5(3):223-234.

Collet C, Adler N, Schwitzguebel J-P & Peringer P (2004). Hydrogen production by *Clostridium thermolactum* during continuous fermentation of lactose. Int J Hydrogen Energy 29:1479-1485.

Cornet JF, Favier L & Dussap CG (2003). Modeling stability of photoheterotrophic continuous cultures in photobioreactors. Biotechnol Prog 19:1216-1227.

Cournac L, Guedeney G, Peltier G & Vignais PM (2004). Sustained photoevolution of molecular hydrogen in a mutant of *Synechocystis* sp. strain PCC 6803 deficient in the type I NADPH-dehydrogenase complex. J Bacteriol 186:1737–1746.

Dalton H (2005). The Leeuwenhoek Lecture 2000 The natural and unnatural history of methane-oxidizing bacteria. Phil Trans R Soc B 360(1458):1207-1222.

Das D & Veziroglu TN (2001). Hydrogen production by biological processes: a survey of literature. Int J Hydrogen Energy 26:13-28.

Davila-Vazquez G, Arriaga S, Alatriste-Mondragón F, de León-Rodríguez A, Rosales-Colunga LM & Razo-Flores E (2008a). Fermentative biohydrogen production: trends and perspectives. Rev Environ Sci Biotechnol 7(1):27-45.

Davila-Vazquez G, Alatriste-Mondragón F, de León Rodríguez A & Razo-Flores E (2008b). Fermentative hydrogen production in batch experiments using lactose, cheese whey and glucose: Influence of initial substrate concentration and pH. Int J Hydrogen Energy 33(19):4989-4997.

Dawson L & Boopathy R (2007). Use of post-harvest sugarcane residue for ethanol production. Bioresour Technol 98(9):1695-1699.

de Vrije T & Claassen PAM (2003). Dark hydrogen fermentations. Bio-methane & Bio-hydrogen. Reith JH, Wijffels RH & Barten H, (Eds). Petten, Netherlands, Dutch Biological Hydrogen Foundation**:** 103-123.

de Vrije T, Mars AE, Budde MAW, Lai MH, Dijkema C, de Waard P & Claassen PAM (2007). Glycolytic pathway and hydrogen yield studies of the extreme thermophile *Caldicellulosiruptor saccharolyticus*. Appl Microbiol Biotechnol 74:1358-1367.

Dien BS, Nichols NN, O'Bryan PJ & Bothast RJ (2000). Development of new ethanologenic *Escherichia coli* strains for fermentation of lignocellulosic biomass. Appl Biochem Biotechnol 84-6:181-196.

Dutta D, De D, Chaudhuri S & Bhattacharya SK (2005). Hydrogen production by cyanobacteria. Microb Cell Fact 4:36.

Easterly JL & Burnham M (1996). Overview of biomass and waste fuel resources for power production. Biomass Bioenergy 10(2-3):79-92.

Eiteman MA & Altman E (2006). Overcoming acetate in *Escherichia coli* recombinant protein fermentations. Trends Biotechnol 24(11):530-536.

Emanuelsson EAC, Arcangeli JP & Livingston AG (2003). The anoxic extractive membrane bioreactor. Water Res 37(6):1231-1238.

Ensign JC (1977). Biomass production from animal wastes. Microbial Energy Conversion. Schlegel HG & Barnea J, (Eds). Oxford, Pergamon**:** 455-483.

Eroğlu E, Gündüz U, Yücel M, Türker L & Eroğlu I (2004). Photobiological hydrogen production by using olive mill wastewater as a sole substrate source. Int J Hydrogen Energy 29:163-171.

Eroğlu E, Eroğlu I, Gündüz U, Türker L & Yücel M (2006). Biological hydrogen production from olive mill wastewater with two-stage processes. Int J Hydrogen Energy 31(11):1527-1535.

Eroğlu I, Aslan K, Gündüz U, Yücel M & Türker L (1997). Continuous hydrogen production by *R. sphaeroides* O.U. 001. International Conference on Biological Hydrogen Production, Waikoloa, Hawaii, Plenum Press, New York.

Eroğlu I, Tabanoğlu A, Gündüz U, Eroğlu E & Yücel M (2008). Hydrogen production by *Rhodobacter sphaeroides* O.U.001 in a flat plate solar bioreactor. Int J Hydrogen Energy 33:531-541.

Fang HHP, Liu H & Zhang T (2005). Phototrophic hydrogen production from acetate and butyrate in wastewater. Int J Hydrogen Energy 30:785-793.

Fang HHP, Li C & Zhang T (2006). Acidophilic biohydrogen production from rice slurry. Int J Hydrogen Energy 31:683-692.

Fascetti E, D'Addario E, Todini O & Robertiello A (1998). Photosynthetic hydrogen evolution with volatile organic acids derived from the fermentation of source selected municipal solid wastes. Int J Hydrogen Energy 23(9):753-760.

Ferchichi M, Crabbe E, Hintz W, Gil GH & Almadidy A (2005). Influence of culture parameters on biological hydrogen production by *Clostridium saccharoperbutylacetonicum* ATCC 27021. World J Microbiol Biotechnol 21(6-7):855-862.

Filatova LV, Berg IA, Krasil'nikova EN, Tsygankov AA, Laurinavichene TV & Ivanovskii RN (2005a). A study of the mechanism of acetate assimilation in purple nonsulfur bacteria lacking the glyoxylate shunt: Acetate assimilation in *Rhodobacter sphaeroides*. Microbiol 74(3):265-269.

Filatova LV, Berg IA, Krasil'nikova EN & Ivanovskii RN (2005b). A study of the mechanism of acetate assimilation in purple nonsulfur bacteria lacking the glyoxylate shunt: Enzymes of the citramalate cycle in *Rhodobacter sphaeroides*. Microbiol 74(3):270-278.

Filho PA & Badr O (2004). Biomass resources for energy in north-eastern Brazil. Appl Energy 77:51-67.

Fissler J, Kohring GW & Giffhorn F (1995). Enhanced hydrogen production from aromatic acids by immobilized cells of *Rhodopseudomonas palustris*. Appl Microbiol Biotechnol 44:43-46.

Franchi E, Tosi C, Scolla G, Penna GD, Rodriguez F & Pedroni PM (2004). Metabolically engineered *Rhodobacter sphaeroides* RV strains for improved biohydrogen photoproduction combined with disposal of food wastes. Mar Biotechnol 6:552-565.

Fuji T, Tarusawa M, Miyanaga M, Kiyota S, Watanabe T & Yabuki M (1987). Hydrogen production from alcohols, malate and mixed electron donors by *Rhodopseudomonas* sp. No. 7. Agric Biol Chem 51(1):1-7.

Ghirardi ML, King PW, Posewitz MC, Maness PC, Fedorov A, Kim K, Cohen J, Schulten K & Seibert M (2005). Approaches to developing biological H_2-photoproducing organisms and processes. Biochem Soc Trans 33:70-72.

Goldemberg J (2007). Ethanol for a sustainable energy future. Science 315:808-810.

Gosse JL, Engel BJ, Rey FE, Harwood CS, Scriven LE & Flickinger MC (2007). Hydrogen production by photoreactive nanoporous latex coatings of nongrowing *Rhodopseudomonas palustris* CGA009. Biotechnol Prog 23(1):124-130.

Grosse S, Laramee L, Wendlandt K-D, McDonald IR, Miguez CB & Kleber H-P (1999). Purification and characterization of the soluble methane monooxygenase of the type II methanotrophic nacterium *Methylocystis* sp. strain WI 14. Appl Environ Microbiol 65(9):3929–3935.

Gübitz GM, Mittelbach M & Trabi M (1999). Exploitation of the tropical oil seed plant *Jatropha curcas* L. Bioresour Technol 67:73-82.

Hallenbeck PC & Benemann JR (2002). Biological hydrogen production; fundamentals and limiting processes. Int J Hydrogen Energy 27(11-12):1185-1193.

Hallenbeck PC (2005). Fundamentals of the fermentative production of hydrogen. Water Sci Technol 52(1-2):21-29.

Happe T, Schutz K & Bohme H (2000). Transcriptional and mutational analysis of the uptake hydrogenase of the filamentous cyanobacterium *Anabaena variabilis* ATCC 29413. J Bacteriol 182(6):1624-31.

Haq Z & Easterly JL (2006). Agricultural residue availability in the United States. Appl Biochem Biotechnol 129-132:3-21.

Hassan MA, Shirai Y, Kusubayashi N, Karim MIA, Nakanishi K & Hashimoto K (1997). The production of polyhydroxyalkanoate from anaerobically treated palm oil mill effluent by *Rhodobacter sphaeroides*. J Ferment Bioeng 83(5):485-488.

Hawkes FR, Dinsdale R, Hawkes DL & Hussy I (2002). Sustainable fermentative hydrogen production: challenges for process optimisation. Int J Hydrogen Energy 27:1339-1347.

Hawkes FR, Hussy I, Kyazze G, Dinsdale R & Hawkes DL (2007). Continuous dark fermentative hydrogen production by mesophilic microflora: Principles and progress. Int J Hydrogen Energy 32:172-184.

Hawkes FR, Forsey H, Premier GC, Dinsdale RM, Hawkes DL, Guwy AJ, Maddy J, Cherryman S, Shine J & Auty D (2008). Fermentative production of hydrogen from a wheat flour industry co-product. Bioresour Technol 99:5020-5029.

Hill J, Nelson E, Tilman D, Polasky S & Tiffany D (2006). Environmental, economic, and energetic costs and benefits of biodiesel and ethanol biofuels. Proc Nat Acad Sci USA 103:11206-11210.

Hillmer P & Gest H (1977a). H_2 metabolism in the photosynthetic bacterium *Rhodopseudomonas capsulata*: H_2 production by growing cultures. J Bacteriol 129(2):724-731.

Hillmer P & Gest H (1977b). H_2 metabolism in the photosynthetic bacterium *Rhodopseudomonas capsulata*: Production and utilisation of H_2 by resting cells. J Bacteriol 129(2):732-739.

Hoekema S, Bijmans M, Janssen M, Tramper J & Wijffels RH (2002). A pneumatically agitated flat-panel photobioreactor with gas re-circulation: anaerobic photoheterotrophic cultivation of a purple non-sulfur bacterium. Int J Hydrogen Energy 27(11-12):1331-1338.

Hoekema S, Douma RD, Janssen M, Tramper J & Wijffels RH (2006). Controlling light-use by *Rhodobacter capsulatus* continuous cultures in a flat-panel photobioreactor. Biotechnol Bioeng 95(4):613-26.

Holmes B & Jones N (2003). Brace yourself for the end of cheap oil. New Sci 179(2406):9.

Hustede E, Steinbuchel A & Schlegel HG (1993). Relationship between the photoproduction of hydrogen and the accumulation of PHB in nonsulfur purple bacteria. Appl Microbiol Biotechnol 39(1):87-93.

Ike A, Toda N, Murakawa T, Hirata K & Miyamoto K (1997). Hydrogen photoproduction from starch in CO_2-fixing microalgal biomass by a halotolerant bacterial community. Biohydrogen, Hawaii, Plenum Press, New York and London.

Ike A, Kawaguchi H, Hirata K & Miyamoto K (2001). Hydrogen photoproduction from starch in algal biomass. Biohydrogen II : An Approach to Environmentally Acceptable Technology. Miyake J, Matsunaga T & San Pietro A, (Eds). Pergamon**:** 53-61.

Ikuta Y, Akano T, Shioji N & Maeda I (1997). Hydrogen production by photosynthetic microorganisms. International Conference on Biological Hydrogen Production, Waikoloa, Hawaii, Plenum Press, New York.

Ivanovskii RN, Krasil'nikova EN & Berg IA (1997). The mechanism of acetate assimilation in the purple nonsulfur bacterium *Rhodospirillum rubrum* lacking isocitrate lyase. Microbiol 66(6):621-626.

Jahn A, Keuntje B, Dorffler M, Klipp W & Oelze J (1994). Optimizing photoheterotrophic H_2 production by *Rhodobacter capsulatus* upon interposon mutagenesis in the *hupL* gene. Appl Microbiol Biotechnol 40:687-690.

Jo JH, Lee DS & Park JM (2006). Modeling and optimization of photosynthetic hydrogen gas production by green alga Chlamydomonas reinhardtii in sulfur-deprived circumstance. Biotechnol Prog 22(2):431-437.

Kadar Z, de Vrije T, van Noorden GE, Budde MAW, Szengyel Z, Reczey K & Claassen PAM (2004). Yields from glucose, xylose, and paper sludge hydrolysate during hydrogen production by the extreme thermophile *Caldicellulosiruptor saccharolyticus*. Appl Biochem Biotechnol 113-116:497-508.

Karlsson A, Vallin L & Ejlertsson J (2008). Effects of temperature, hydraulic retention time and hydrogen extraction rate on hydrogen production from the fermentation of food industry residues and manure. Int J Hydrogen Energy 33(3):953-962.

Kataoka N, Miya A & Kiriyama K (1997). Studies on hydrogen production by continuous culture system of hydrogen-producing anaerobic bacteria. Water Sci Technol 36(6-7):41-47.

Katsuda T, Arimoto T, Igarashi K, Azuma M, Kato J, Takakuwa S & Ooshima H (2000). Light intensity distribution in the externally illuminated cylindrical photo-bioreactor and it's application to hydrogen production by *Rhodobacter capsulatus*. Biochem Eng J 5:157-164.

Kawaguchi H, Hashimoto K, Hirata K & Miyamoto K (2001). H_2 production from algal biomass by a mixed culture of *Rhodobium marinum* A-501 and *Lactobacillus amylovorus*. J Biosci Bioeng 91(3):277-282.

Keeling CD & Whorf TP (2005). Atmospheric CO_2 records from sites in the SIO air sampling network. Trends: A Compendium of Data on Global Change. Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory, U.S. Department of Energy, Oak Ridge, Tenn., U.S.A.

Kern M, Koch HG & Klemme JH (1992). EDTA activation of H_2 photoproduction by *Rhodospirillum rubrum*. Appl Microbiol Biotechnol 37(4):496-500.

Khatipov E, Miyake M, Miyake J & Asada Y (1998). Accumulation of poly-β-hydroxybutyrate by *Rhodobacter sphaeroides* on various carbon and nitrogen substrates. FEMS Microbiol Lett 162:39-45.

Kim E-J, Kim J-S, Kim M-S & Lee JK (2006a). Effect of changes in the level of light harvesting complexes of *Rhodobacter sphaeroides* on the photoheterotrophic production of hydrogen. Int J Hydrogen Energy 31:531-538.

Kim M-S, Lee TJ, Yoon YS, Lee IG & Moon KW (2001). Hydrogen production from food processing wastewater and sewage sludge by anaerobic dark fermentation combined with photo-fermentation. Biohydrogen II : An Approach to Environmentally Acceptable Technology. Miyake J, Matsunaga T & San Pietro A, (Eds). Pergamon**:** 263-272.

Kim M-S, Baek J-S & Lee JK (2006b). Comparison of H_2 accumulation by *Rhodobacter sphaeroides* KD131 and its uptake hydrogenase and PHB synthase deficient mutant. Int J Hydrogen Energy 31:121-127.

Kim M-S, Baek J-S, Yun Y-S, Sim SJ, Park S & Kim S-C (2006c). Hydrogen production from *Chlamydomonas reinhardtii* biomass using a two-step conversion process: Anaerobic conversion and photosynthetic fermentation. Int J Hydrogen Energy 31:812-816.

Kim NJ, Lee JK & Lee CJ (2004). Pigment reduction to improve photosynthetic productivity of *Rhodobacter sphaeroides*. J Gen Microbiol 1692(28):607-16.

Kim S-H, Han S-K & Shin H-S (2004). Feasibility of biohydrogen production by anaerobic co-digestion of food waste and sewage sludge. Int J Hydrogen Energy 29:1607-1616.

Kirkpatrick C, Maurer LM, Oyelakin NE, Yoncheva YN, Maurer R & Slonczewski JL (2001). Acetate and formate stress: Opposite responses in the proteome of *Escherichia coli*. J Bacteriol 183(21):6466-6477.

Knappe J (1987). Anaerobic dissimilation of pyruvate. *Escherichia coli* and *Salmonella Typherium*. Niedhardt FC, (Ed.). Washington DC, Am Soc Microbiol. 1**:** 151-155.

Ko IB & Noike T (2002). Use of blue optical filters for suppression of growth of algae in hydrogen producing non-axenic cultures of *Rhodobacter sphaeroides* RV. Int J Hydrogen Energy 27(11-12):1297-1302.

Koku H, Eroğlu I, Gündüz U, Yücel M & Türker L (2002). Aspects of the metabolism of hydrogen production by *Rhodobacter sphaeroides*. Int J Hydrogen Energy 27:1315-1329.

Kondo T, Arakawa M, Wakayama T & Miyake J (2002). Hydrogen production by combining two types of photosynthetic bacteria with different characteristics. Int J Hydrogen Energy 27(11-12):1303-1308.

Kondo T, Wakayama T & Miyake J (2006). Efficient hydrogen production using a multi-layered photobioreactor and a photosynthetic bacterium mutant with reduced pigment. Int J Hydrogen Energy 31(11):1522-1526.

Kraemer JT & Bagley DM (2007). Improving the yield from fermentative hydrogen production. Biotechnol Lett 29:685-695.

Kurokawa T & Shigeharu T (2005). Effects of formate on fermentative hydrogen production by *Enterobacter aerogenes*. Mar Biotechnol 7:112-118.

Kvaalen E, Wankat PC & McKenzie BA (2006). Alcohol distillation: Basic principles, equipment, performance relationships, and safety. www.ces.purdue.edu/extmedia/AE/AE-117.html, accessed May 2008.

Kyazze G, Dinsdale R, Guwy AJ, Hawkes FR, Premier GC & Hawkes DL (2007). Performance characteristics of a two-stage dark fermentative system producing hydrogen and methane continuously. Biotechnol Bioeng 97(4):759-770.

Larminie J & Dicks A (2003). Fuel cell systems explained, 2nd ed., John Wiley & Sons, Chichester, Sussex, UK.

Laurinavichene TV, Fedorov AS, Ghirardi ML, Siebert M & Tsygankov AA (2006). Demonstration of sustained hydrogen production by immobilised, sulfur-deprived *Chlamydomonas reinhardtii* cells. Int J Hydrogen Energy 31:659-667.

Lee CM, Chen PC, Wang CC & Tung YC (2002). Photohydrogen production using purple nonsulfur bacteria with hydrogen fermentation reactor effluent. Int J Hydrogen Energy 27(11-12):1309-1313.

Levin DB, Pitt L & Love M (2004a). Biohydrogen production: prospects and limitations to practical application. Int J Hydrogen Energy 29:173-185.

Levin DB (2004b). Re: Biohydrogen production: prospects and limitations to practical application-Erratum. Int J Hydrogen Energy 29:1425-1426.

Levin DB, Islam R, Cicek N & Sparling R (2006). Hydrogen production by *Clostridium thermocellum* 27405 from cellulosic biomass substrates. Int J Hydrogen Energy 31(11):1496-1503.

Levin DB, Zhu H, Beland M, Cicek N & Holbein BE (2007). Potential for hydrogen and methane production from biomass residues in Canada. Bioresour Technol 98:654-660.

Li H, Mustacci R, Knowles CJ, Skibar W, Sunderland G, Dalrymple I & Jackman S, A. (2004). An electrokinetic bioreactor: using direct electric current for enhanced lactic acid fermentation and product recovery. Tetrahed 60:655-661.

Liang TM, Cheng SS & Wu KL (2002). Behavioral study on hydrogen fermentation reactor installed with silicone rubber membrane. Int J Hydrogen Energy 27(11-12):1157-1165.

Liessens J & Verstraete W (1986). Selective inhibitors for continuous non-axenic hydrogen production by *Rhodobacter capsulatus*. J Appl Bacteriol 61(6):547-557.

Lin C-Y & Lay CH (2005). A nutrient formation for fermentative hydrogen production using anaerobic sewage sludge microflora. Int J Hydrogen Energy 30:285-292.

Lin Y & Tanaka S (2006). Ethanol fermentation from biomass resources: current state and prospects. Appl Microbiol Biotechnol 69(6):627-642.

Lindblad P, Christensson K, Lindberg P, Fedorov A, Pinto F & Tsygankov A (2002). Photoproduction of H_2 by wildtype *Anabaena* PCC 7120 and a hydrogen uptake deficient mutant: From laboratory experiments to outdoor culture. Int J Hydrogen Energy 27(11-12):1271-1281.

Ma F & Hanna MH (1999). Biodiesel production: a review. Bioresour Technol 70:1-15.

Mabee WE, Fraser EDG, McFarlane PN & Saddler JN (2006). Canadian biomass reserves for biorefining. Appl Biochem Biotechnol 129-132:22-40.

Macaskie LE (2004). Biological hydrogen production from crops & sugar wastes. Final Report, EPSRC Project GR/S62406/01.

Macaskie LE, Baxter-Plant VS, Creamer NJ, Humphries AC, Mikheenko IP, Mikheenko PM, Penfold DW & Yong P (2005). Applications of bacterial hydrogenases in waste decontamination, manufacture of novel bionanocatalysts and in sustainable energy. Biochem Soc Trans 33(Pt 1):76-9.

Macedo IC, Leal MRLV & Silva JEAR (2004). Assessment of greenhouse gas emissions in the production and use of fuel ethanol in Brazil, available at www.unica.com.br/i_pages/files/gee3.pdf.

Macler BA, Pelroy RA & Bassham JA (1979). Hydrogen formation in nearly stoichiometric amounts from glucose by a *Rhodopseudomonas* mutant. J Bacteriol 138(2):446-452.

Macler BA & Bassham JA (1988). Carbon allocation in wild-type and Glc^+ *Rhodobacter sphaeroides* under photoheterotrophic conditions. Appl Environ Microbiol 54(11):2737-2741.

Madamwar D, Garg N & Shah V (2000). Cyanobacterial hydrogen production. World J Microbiol Biotechnol 16:757-767.

Mandal B, Nath K & Das D (2006). Improvement of biohydrogen production under decreased partial pressure of H_2 by *Enterobacter cloacae*. Biotechnol Lett 28:831-835.

Martínez-Herrera J, Siddhuraju P, Francis G, Dávila-Ortíz G & Becker K (2006). Chemical composition, toxic/antimetabolic constituents, and effects of different treatments on their levels, in four provenances of *Jatropha curcas* L. from Mexico. Food Chem 96:80-89.

Masukawa H, Mochimaru M & Sakurai H (2002). Disruption of the uptake hydrogenase gene, but not of the bidirectional hydrogenase gene, leads to enhanced photobiological hydrogen production by the nitrogen-fixing cyanobacterium *Anabaena* sp. PCC 7120. Appl Microbiol Biotechnol 58(5):618-24.

McCormick DB (1998). Oxidation–reduction reactions. Encyclopedia of Life Sciences. Chichester, John Wiley & Sons, Ltd.

Melis A & Happe T (2004). Trails of green alga hydrogen research - from Hans Gaffron to new frontiers. Photosynth Res 80(1-3):401-409.

Melis A & Melnicki MR (2006). Integrated biological hydrogen production. Int J Hydrogen Energy 31(11):1563-1573.

Miura Y, Ohta S, Mano M & Miyamoto K (1986). Isolation and characterisation of a unicellular green alga exhibiting high activity in dark hydrogen production. Agric Biol Chem 50(11):2837-2844.

Miura Y, Saitoh C, Matsuoka S & Miyamoto K (1992). Stably sustained hydrogen production with high molar yield through a combination of a marine green alga and a photosynthetic bacterium. Biosci Biotechnol Biochem 56(5):751-754.

Miyake J, Mao X-Y & Kawamura S (1984). Photoproduction of hydrogen from glucose by a co-culture of a photosynthetic bacterium and *Clostridium butyricum*. J Ferment Technol 62(6):531-535.

Miyake J, Miyake M & Asada Y (1999). Biotechnological hydrogen production: research for efficient light conversion. J Biotechnol 70:89-101.

Miyamoto K, Ohta S, Nawa Y, Mori Y & Miura Y (1987). Hydrogen production by a mixed culture of a green alga C*hlamydomonas reinhardtii* and a photosynthetic bacterium *Rhodospirillum rubrum*. Agric Biol Chem 51(5):1391-1324.

Mizuno O, Dinsdale R, Hawkes FR, Hawkes DL & Noike T (2000). Enhancement of hydrogen production from glucose by nitrogen gas sparging. Bioresour Technol 73:59-65.

Nakada E, Asada Y, Arai T & Miyake J (1995). Light penetration into cell suspensions of photosynthetic bacteria and relation to hydrogen production. J Ferment Bioeng 80(1):53-57.

Nandi R & Sengupta S (1998). Microbial production of hydrogen: An overview. Crit Rev Microbiol 24(1):61-84.

Nastari PM, Macedo IC & Szwarc A (2005). Observations on the draft document entitled "Potential for Biofuels for transport in developing countries". The World Bank, Air Quality Thematic Group, p. 8. http://www.unica.com.br/i_pages/files/ibm.pdf.

Nath K & Das D (2003). Hydrogen from biomass. Curr Sci 85(3):265-271.

Nath K & Das D (2004a). Biohydrogen production as a potential energy source - Present state-of-art. J Sci Indust Res 63(9):729-738.

Nath K & Das D (2004b). Improvement of fermentative hydrogen production: various approaches. Appl Microbiol Biotechnol 65(5):520-529.

Nath K, Kumar A & Das D (2005). Hydrogen production by *Rhodobacter sphaeroides* strain O.U.001 using spent media of *Enterobacter cloacae* strain DM11. Appl Microbiol Biotechnol 68:533-541.

Nath K, Muthukumar M, Kumar A & Das D (2008). Kinetics of two-stage fermentation process for the production of hydrogen. Int J Hydrogen Energy 33(4):1195-1203.

Nielsen AM, Amandusson H, Bjorklund R, Dannetun H, Ejlertsson J, Ekedahl L-G, Lundstrom I & Svensson BH (2001). Hydrogen production from organic waste. Int J Hydrogen Energy 26:547-550.

Nowak M (2004). Urban Agriculture on the Rooftop, Cornell University, available at www3.telus.net/public/a6a47567/roofgarden_thesis.pdf.

Odom JM & Wall JD (1983). Photoproduction of H_2 from cellulose by an anerobic bacterial culture. Appl Environ Microbiol 45(4):1300-1305.

Oh SE & Logan BE (2005). Hydrogen and electricity production from a food processing wastewater using fermentation and microbial fuel cell technologies. Water Res 39(19):4673-4682.

Oh Y-K, Seol E-H, Kim M-S & Park S (2004). Photoproduction of hydrogen from acetate by a chemoheterotrophic bacterium *Rhodopseudomonas palustris* P4. Int J Hydrogen Energy 29:1115-1121.

Ordal EJ & Halvorson HO (1939). A comparison of hydrogen production from sugars and formic acid by normal and variant strains of *Escherichia coli*. J Bacteriol 38:199-220.

Öztürk Y, Yücel M, Daldal F, Mandaci S, Gündüz U, Türker L & Eroğlu I (2006). Hydrogen production by using *Rhodobacter capsulatus* mutants with genetically modified electron transfer chains. Int J Hydrogen Energy 31(11):1545-1552.

Park W, Hyun SH, Oh SE, Logan BE & Kim IS (2005). Removal of headspace CO_2 increases biological hydrogen production. Environ Sci Technol 39(12):4416-4420.

Penfold DW, Forster CF & Macaskie LE (2003). Increased hydrogen production by *Escherichia coli* strain HD701 in comparison with the wild-type parent strain MC4100. Enz Microb Technol 33(2-3):185-189.

Pimentel D (2001). The limitations of biomass energy. in Encyclopedia of Physical Science and Technology, 3rd edition. Meyers R, (Ed.), Academic Press, San Diego, CA**:** p.159-171.

Polle JEW, Kanakagiri S, Jin E, Masuda T & Melis A (2002). Truncated chlorophyll antenna size of the photosystems - a practical method to improve microalgal productivity and hydrogen production in mass culture. Int J Hydrogen Energy 27(11-12):1257-1264.

Redwood MD & Macaskie LE (2006). A two-stage, two-organism process for biohydrogen from glucose. Int J Hydrogen Energy 31(11):1514-1521.

Redwood MD & Macaskie LE (2007a). Method and apparatus for biohydrogen production. British Patent Application No. 0705583.3. UK

Redwood MD & Macaskie LE (2007b). Efficient bio-H_2 and PEM-FC catalyst. Proceedings of the 7th Hydrogen - Power and Theoretical Engineering Solutions International Symposium (HyPoThESIS VII), Merida, Mexico. CICY ISBN:968-6114-21-1.

Redwood MD, Mikheenko IP, Sargent F & Macaskie LE (2007c). Dissecting the roles of *E. coli* hydrogenases in biohydrogen production. FEMS Microbiol Lett 278:48-55.

Ren N, Li J, Wang Y & Liu S (2006). Biohydrogen production from molasses by anaerobic fermentation with a pilot-scale bioreactor system. Int J Hydrogen Energy 31:2147-2157.

Rocha JS, Barbosa MJ & Wijffels RH (2001). Hydrogen production by photoheterotrophic bacteria: Culture media, yields and efficiencies. Biohydrogen II : An Approach to Environmentally Acceptable Technology. Miyake J, Matsunaga T & San Pietro A, (Eds). Pergamon**:** 3-32.

Roe AJ, McLaggan D, Davidson I, O'Byrne C & Booth IR (1998). Perturbation of anion balance during inhibition of growth of *Echerichia coli* by weak acids. J Bacteriol 180(4):767-722.

Rogers PL, Jeon YJ & Svenson CJ (2005). Application of biotechnology to industrial sustainability. Trans IChemE Part B 83(B6):499-503.

Sakurai H & Masukawa H (2007). Promoting R & D in photobiological hydrogen production utilizing mariculture-raised cyanobacteria. Mar Biotechnol 9:128-145.

Sasikala C, Ramana CV & Prasad GS (1994a). H_2 production by mixed cultures. World J Microbiol Biotechnol 10(2):221-223.

Sasikala C, Ramana CV & Rao PR (1994b). Nitrogen fixation by *Rhodopseudomonas palustris* OU 11 with aromatic compounds as carbon source / electron donors. FEMS Microbiol Lett 122:75-78.

Sasikala K, Ramana CV, Rao PR & Subrahmanyam M (1990). Effect of gas phase on the photoproduction of hydrogen and substrate conversion efficiency on the photosynthetic bacterium *Rhodobacter sphaeroides* O.U. 001. Int J Hydrogen Energy 15(11):795-797.

Sasikala K, Ramana CV & Subrahmanyam M (1991). Photoproduction of hydrogen from wastewater of a lactic acid fermentation plant by a purple non-sulfur photosynthetic bacterium *Rhodobacter sphaeroides*. Indian J Exp Biol 29(74-75).

Sasikala K, Ramana CV & Rao PR (1992). Photoproduction of hydrogen from the waste water of a distillery by *Rhodobacter sphaeroides* O.U. 001. Int J Hydrogen Energy 17(1):23-27.

Sasikala K, Ramana CV, Rao PR & Kovacs KL (1995). Anoxygenic Phototrophic Bacteria : Physiology and Advances in Hydrogen Production Technology. Adv Appl Microbiol 38:211-295.

Sauter M, Bohm R & Bock A (1992). Mutational analysis of the operon (*hyc*) determining hydrogenase-3 formation in *Escherichia coli*. Mol Microbiol 6(11):1523-1532.

Schnoor JL (2006). Biofuels and the environment. Environ Sci Technol 40(13):4024.

Shi X-Y & Yu Q-H (2006). Continuous production of hydrogen from mixed volatile fatty acids with *Rhodopseudomonas capsulata*. Int J Hydrogen Energy 31:1641-1647.

Sode K, Watanabe M, Makimoto H & Tomiyama M (1999). Construction and characterisation of fermentative lactate dehydrogenase *E. coli* mutant and its potential for bacterial hydrogen production. Appl Biochem Biotechnol 77-79:317-323.

Sode K, Yamamoto S & Tomiyama M (2001). Metabolic engineering approaches for the improvement of bacterial hydrogen production based on *Escherichia coli* mixed acid fermentation. Biohydrogen II : An Approach to Environmentally Acceptable Technology. Miyake J, Matsunaga T & San Pietro A, (Eds). Pergamon**:** 195-204.

Splendiani A, Nicolella C & Livingston AG (2003). A novel biphasic extractive membrane bioreactor for minimization of membrane-attached biofilms. Biotechnol Bioeng 83(1):8-19.

Srivastava A & Prasad R (2000). Triglycerides-based diesel fuels. Renew Sustain Energy Rev 4(2):111-133.

Staubmann R, Foidl G, Foidl N, Guebitz GM, Lafferty R, Valencia-Arbizu VM & Walter S (1997). Biogas production from *Jatropha curcas* press-cake. Appl Biochem Biotechnol 63-65(0):457-467.

Stephenson M & Stickland LH (1932). Hydrogenlyases: Bacterial enzymes liberating molecular hydrogen. Bacteriol J 26:712-724.

Stern N (2006). Stern review executive summary. New Economics Foundation. Available at news.bbc.co.uk/1/shared/bsp/hi/pdfs/30_10_06_exec_sum.pdf.

Tabita FR (1995). The biochemistry and metabolic regulation of carbon metabolism and CO_2 fixation in purple bacteria. Anoxygenic Photosynthetic Bacteria. Blankenship RE, Madigan MT & Bauer CE, (Eds). 885-914.

Taillez P, Girard H, Longin R, Beguin P & Millet J (1983). Cellulose fermentation by an asporogenic mutant and an ethanol-tolerant mutant of *Clostridium thermocellum*. Appl Environ Microbiol 55(1):203-206.

Tamagnini P, Axelsson R, Lindberg P, Oxelfelt F, Wunschiers R & Lindblad P (2002). Hydrogenases and hydrogen metabolism in cyanobacteria. Microb Mol Biol Rev 66(1):1-20.

Tanisho S & Ishiwata Y (1995). Continuous hydrogen production from molasses by fermentation using urethane foam as a support of flocks. Int J Hydrogen Energy 20(7):541-545.

Tanisho S, Kuromoto M & Kadokura N (1998). Effect of CO_2 removal on hydrogen evolution by fermentation. Int J Hydrogen Energy 23(7):559-563.

Tao Y, Chen Y, Wu Y, He Y & Zhou Z (2007). High hydrogen yield from a two-step process of dark and photo-fermentation of sucrose. Int J Hydrogen Energy 32:200-206.

Tao Y, He Y, Wu Y, Liu F, Li X, Zong W & Zhou Z (2008). Characteristics of a new photosynthetic bacterial strain for hydrogen production and its application in wastewater treatment. Int J Hydrogen Energy 33(3):963-973.

Teplyakov VV, Gassanova LG, Sostina EG, Slepova EV, Modigell M & Netrusov AI (2002). Lab-scale bioreactor integrated with active membrane system for hydrogen production: experience and prospects. Int J Hydrogen Energy 27:1149-1155.

Thangaraj A & Kulandaivelu G (1994). Biological hydrogen photoproduction using dairy and sugarcane wastewaters. Bioresour Technol 48:9-12.

Thauer R (1977). Limitation of microbial H_2-formation via fermentation. Microbial Energy Conversion. Schlegel HG & Barnea J, (Eds). Oxford, Pergamon**:** 201-204.

Tiwari AK, Kumar A & Raheman H (2007). Biodiesel production from jatropha oil (*Jatropha curcas*) with high free fatty acids: An optimized process. Biomass Bioenergy 31:569-575.

Tosatto SCE, Toppo S, Donatella C, Giacometti GM & Costantini P (2008). Comparative analysis of [FeFe] hydrogenase from *Thermotogales* indicates the molecular basis of resistance to oxygen inactivation. Int J Hydrogen Energy 33(2):570-578.

Troshina O, Serebryakova L, Sheremetieva M & Lindblad P (2002). Production of H_2 by the unicellular cyanobacterium *Gloeocapsa alpicola* CALU 743 during fermentation. Int J Hydrogen Energy 27(11-12):1283-1289.

Tsygankov A (2007). Nitrogen-fixing cyanobacteria: A review. Appl Biochem Microbiol 43(3):250.

Tsygankov AA, Fedorov AS, Laurinavichene TV, Gogotov IN, Rao KK & Hall DO (1998). Actual and potential rates of hydrogen photoproduction by continuous culture of the purple non-sulphur bacterium *Rhodobacter capsulatus*. Appl Microbiol Biotechnol 49(1):102-107.

Tsygankov AA (2001). Laboratory scale photobioreactors. Appl Biochem Microbiol 37(4):333-341.

Tsygankov AA, Fedorov AS, Kosourov SN & Rao KK (2002). Hydrogen production by cyanobacteria in an automated outdoor photobioreactor under aerobic conditions. Biotechnol Bioeng 80(7):777-83.

Tsygankov AA (2007). Biological generation of hydrogen. Russ J Gen Chem 77(4):685-693.

Turkarslan S, Yiğit OD, Aslan K, Eroğlu I & Gündüz U (1997). Photobiological hydrogen production by *R. sphaeroides* O.U 001 by utilisation of waste water from milk industry. International Conference on Biological Hydrogen Production, Waikoloa, Hawaii, Plenum Press, New York.

Ueno Y, Kawai T, Sato S, Otsuka S & Morimoto S (1995). Biological production of hydrogen from cellulose by natural anaerobic microflora. J Ferment Bioeng 97(4):395-397.

Valdez-Vazquez I, Sparling R, Risbey D, Rinderknecht-Seijas N & Poggi-Varraldo HM (2005). Hydrogen generation via anaerobic fermentations of paper mill wastes. Bioresour Technol 96:1907-1913.

Valdez-Vazquez I, Rios-Leal E, Carmona-Martinez A, Munoz-Paez KM & Poggi-Varaldo HM (2006). Improvement of biohydrogen production from solid wastes by intermittent venting and gas flushing of batch reactors headspace. Environ Sci Technol 40:3509-3415.

Van Ginkel S & Logan BE (2005). Inhibition of biohydrogen production by undissociated acetic and butyric acids. Environ Sci Technol 39:9350-9356.

Van Groenestijn JW, Hazewinkel JHO, Nienoord M & Bussmann PJT (2002). Energy aspects of biological hydrogen production in high rate bioreactors operated in the thermophilic temperature range. Int J Hydrogen Energy 27(11-12):1141-1147.

Van Haaster DJ, Hagedoorn PL, Jongejan JA & Hagen WR (2005). On the relationship between affinity for molecular hydrogen and the physiological directionality of hydrogenases. Biochem Soc Trans 33(1):12-14.

Van Niel EWJ, Budde MAW, de Haas GG, van der Wal FJ, Claassen PAM & Stams AJM (2002). Distinctive properties of high hydrogen producing extreme thermophiles, *Caldicellulosiruptor saccharolyticus* and *Thermotoga elfii*. Int J Hydrogen Energy 27(11-12):1390-1398.

Van Niel EWJ, Claassen PAM & Stams AJM (2003). Substrate and product inhibition of hydrogen production by the extreme thermophile, *Caldicellulosiruptor saccharolyticus*. Biotechnol Bioeng 81(3):255-262.

Van Ooteghem SA, Jones A, van der Lelie D, Dong B & Mahajan D (2004). H_2 production and carbon utilization by *Thermotoga neapolitana* under anaerobic and microaerobic growth conditions. Biotechnol Lett 26(15):1223-1232.

Vardar-Schara G, Maeda T & Wood TK (2008). Metabolically engineered bacteria for producing hydrogen via fermentation. Microb Technol 1(2):107-125.

Vasilyeva L, Miyake M, Khatipov E, Wakayama T, Sekine M, Hara M, Nakada E, Asada Y & Miyake J (1999). Enhanced hydrogen production by a mutant of *R. sphaeroides* having an altered light-harvesting system. J Biosci Bioeng 87(5):619-624.

Vignais PM, Colbeau M, Willison JC & Jouanneau Y (1985). Hydrogenase, nitrogenase, and hydrogen metabolism in the photosynthetic bacteria. Adv Microb Physiol 26:155-234.

Vignais PM, Billoud B & Meyer J (2001). Classification and phylogeny of hydrogenases. FEMS Microbiol Rev 25(4):455-501.

Vincenzini M, Marchini A, Ena A & DePhilippis R (1997). H_2 and poly-β-hydroxybutyrate, two alternative chemicals from purple non sulfur bacteria. Biotechnol Lett 19(8):759-762.

Wakayama T, Nakada E, Asada Y & Miyake J (2000). Effect of light/dark cycle on bacterial hydrogen production by *Rhodobacter sphaeroides* RV - From hour to second range. Appl Biochem Biotechnol 84-6:431-440.

Wakayama T & Miyake J (2001). Hydrogen from biomass. Biohydrogen II : An Approach to Environmentally Acceptable Technology. Miyake J, Matsunaga T & San Pietro A, (Eds). Pergamon**:** 3-32.

Wakayama T & Miyake J (2002). Light shade bands for the improvement of solar hydrogen production efficiency by *Rhodobacter sphaeroides* RV. Int J Hydrogen Energy 27:1495-1500.

Wall JD & Gest H (1979). Derepression of Nitrogenase Activity in Glutamine Auxotrophs of *Rhodopseudomonas capsulata*. J Bacteriol 137(3):1459-1463.

Weetall HH, Sharma BP & Detar CC (1989). Photometabolic production of hydrogen from organic substrates by free and immobilised mixed cultures of *Rhodospirillum rubrum* and *Klebsiella pneumoniae*. Biotechnol Bioeng 23:605-614.

Willison JC, Madern D & Vignais PM (1984). Increased photoproduction of hydrogen by non-autotrophic mutants of *Rhodopseudomonas capsulata*. Biochem J 219(2):593-600.

Worin NA, Lissolo T, Colbeau A & Vignais PM (1996). Increased H_2 photoproduction by *Rhodobacter capsulatus* strains deficient in uptake hydrogenase. J Mar Biotechnol 4:28-33.

Wykoff DD, Davies JP, Melis A & Grossman AR (1998). The regulation of photosynthetic electron-transport. Plant Physiol 117:129-139.

Yagi K, Maeda I, Idehara K, Miura Y, Akano T, Fukatu K, Ikuta Y & Nakamura HK (1994). Removal of inhibition by ammonium ion in nitrogenase-dependent hydrogen evolution of a marine photosynthetic bacterium, *Rhodopseudomonas* sp strain W-1s. Appl Biochem Biotechnol 45-6:429-436.

Yetis M, Gündüz U, Eroğlu I, Yücel M & Türker L (2000). Photoproduction of hydrogen from sugar refinery wastewater by *Rhodobacter sphaeroides* O.U. 001. Int J Hydrogen Energy 25:1035-1041.

Yiğit OD, Gündüz U, Türker L, Yücel M & Eroğlu I (1999). Identification of by-products in hydrogen producing bacteria; *Rhodobacter sphaeroides* O.U. 001 grown in the waste water of a sugar refinery. J Biotechnol 70:125-131.

Ying Li R, Zhang T & Fang HHP (2008). Characteristics of a phototrophic sludge producing hydrogen from acetate and butyrate. Int J Hydrogen Energy 33(9):2147-2155.

Yokoi H, Mori S, Hirose J, Hayashi S & Takasaki Y (1998). H_2 production from starch by a mixed culture of *Clostridium butyricum* and *Rhodobacter* sp. M-19. Biotechnol Lett 20(9):895-899.

Yokoi H, Saitsu A, Uchida H, Hirose J, Hayashi S & Takasaki Y (2001). Microbial hydrogen production from sweet potato starch residue. J Biosci Bioeng 91(1):58-63.

Yokoi H, Maki R, Hirose J & Hayashi S (2002). Microbial production of hydrogen from starch-manufacturing wastes. Biomass Bioenergy 22:389-395.

Yoon JH, Shin JH, Kim M-S, Sim SJ & Park TH (2006). Evaluation of conversion efficiency of light to hydrogen energy by *Anabaena variabilis*. Int J Hydrogen Energy 31:721 – 727.

Yoshida A, Nishimura T, Kawaguchi H, Inui M & Yukuwa H (2005). Enhanced hydrogen production from formic acid by formate hydrogen lyase-overexpressing *Escherichia coli* strains. Appl Environ Microbiol 71(11):6762-6768.

Yoshino F, Ikeda H, Masukawa H & Sakurai H (2007). High photobiological hydrogen production activity of a Nostoc sp. PCC 7422 uptake hydrogenase-deficient mutant with high nitrogenase activity. Mar Biotechnol (NY) 9(1):101-12.

Yun S-I & Ohta Y (2005). Removal of volatile fatty acids with immobilised *Rhodococcus* sp. B261. Bioresour Technol 96:41-46.

Zacchi G & Axelsson A (1989). Economic evaluation of preconcentration in product and of ethanol from dilute sugar solutions. Biotechnol Bioeng 34:223-233.

Zhu H, Miyake J, Tsygankov AA & Asada Y (1995). Hydrogen production from highly concentrated organic wastewater by photosynthetic bacteria & anaerobic bacteria. Water Treatment 10:61-68.

Zhu H, Suzuki T, Tsygankov AA, Asada Y & Miyake J (1999a). Hydrogen production from tofu wastwater by *Rhodobacter sphaeroides* immobilised in agar gels. Int J Hydrogen Energy 24:305-310.

Zhu H, Wakayama T, Suzuki T, Asada Y & Miyake J (1999b). Entrapment of *Rhodobacter sphaeroides* in cationic polymer/agar gels for hydrogen production in the presence of NH_4^+. J Biosci Bioeng 88(5):507-512.

Zhu H, Wakayama T, Asada Y & Miyake J (2001). Hydrogen production by four cultures with participation by anoxygenic photosynthetic bacterium and anaerobic bacterium in the presence of NH_4^+. Int J Hydrogen Energy 26(11):1149-1154.

Zhu HG, Ueda S, Asada Y & Miyake J (2002). Hydrogen production as a novel process of wastewater treatment - Studies on tofu wastewater with entrapped *R. sphaeroides* and mutagenesis. Int J Hydrogen Energy 27(11-12):1349-1357.

Zinchenko VV, Kopteva AV, Belavina NV, Mitronova TN, Frolova VD & Shestakov SV (1991). The study of *Rhodobacter sphaeroides* mutants of different type with derepressed nitrogenase. Genetika 27(6):991-999.

Zinchenko VV, Babykin M, Glaser V, Mekhedov S & Shestakov SV (1997). Mutation in *ntrC* gene leading to the derepression of nitrogenase synthesis in *Rhodobacter sphaeroides*. FEMS Microbiol Lett 147:57-61.

Zurrer H & Bachofen R (1979). Hydrogen production by the photosynthetic bacterium *Rhodospirillum rubrum*. Appl Environ Microbiol 37(5):789-793.

Züttel A (2004). Hydrogen storage methods. Naturwissenschaften 91(4):157-172.

Table 1 : Summary of dual systems employed for bio-hydrogen production

Feeds, substrates, supplements	1st stage		Integration strategy	2nd stage		Overall productivity	Notes, limitations, caveats	Source
	Inoculum and mode	Productivity		Organism and mode	Productivity			
Microalgae – Anoxygenic photosynthetic bacteria								
CO_2 (sole C source)	*Chlamydomonas* MGA161 Batch	*ca.* 1.3 mol H_2 / hexose + acetate and ethanol	Sequential batch-transfer	Photosynthetic bacterium W-1S Fed-batch	*ca.* 6.7 mol H_2/mol hexose	8 mol H_2/mol hexose for (7 d) [a]	12 h day/night cycle	(Miura et al. 1992)
					ca. 9.2 mol H_2/mol hexose	10.5 mol H_2/mol hexose (7 d) [a]	Continuous illumination	
CO_2, NG	*Chlamydomonas* sp. strain MGA161 Repeated batch, 30 °C	Av. 24.4 mmol hexose/d 80% conversion to glycerol, acetate, ethanol	Sequential batch-transfer	*Rhodovulum sulfidophilum* Purple sulfur bacteria Fed-batch	Average: 3.4 L H_2/d	5.8 mol H_2/mol hexose [b] 23 d operation	Pilot scale. Difficulty with contamination of the 2nd stage	(Akano et al. 1996; Ikuta et al. 1997)
NG	*C. reinhardtii*, batch	NG	Co-culture ratio NG	*Rhodospirillum rubrum* PNS bacteria batch	NG	NG	Qualitative success, data NG	(Melis & Melnicki 2006)
Cyanobacteria – Anoxygenic photosynthetic bacteria								
Glucose	*Synechoccus cedrorum* batch	mol H_2 / mol hexose free: 0.013 immob. : 0.01	Co-culture 1:1 (vol)	*Rhodobacter sphaeroides* O.U.001 PNS bacteria batch	mol H_2/mol hexose free: 0.186 immob. : 3.82	mol H_2/mol hexose free: 0.702 [a] immob. : 0	Continuous illumination (2.4 klux)	(Sasikala et al. 1994a)
SOT medium lacking nitrate	*Spirulina platensis* Batch N-starvation	Light phase: 1.03 mmol hexose/L/d Dark phase: 1 hexose → 0.68 H_2 + 0.4 acetate + 0.15 formate	Sequential batch-transfer	*R. sphaeroides* RV PNS bacteria batch	Nearly stoichiometric	*ca.* 2 mol H_2/mol hexose [b] 2 mmol H_2/day/L	Light-dark cycle (72 h light, 24 h dark)	(Aoyama et al. 1997)

Obligate anaerobic fermenters – Anoxygenic photosynthetic bacteria								
glucose	*Clostridium butyricum* batch	16 % of total H_2 1.1 mol H_2/mol hexose [a]	Immobilised co-culture 1:5 (mass)	*R. sphaeroides* RV PNS bacteria batch	84 % of total H_2 [a] *est.* 70.4 % efficiency [b]	7.0 mol H_2 mol hexose [a]	Continuous illumination, > 300 h	(Miyake et al. 1984)
Tofu or alcohol wastewater	*C. paraputrificum* Batch, 30 ºC	mL/h/L 10 % Tofu: 68 50 % Alcohol: 90	Sequential batch-transfer	*R. sphaeroides* RV PNS bacteria batch	µmol/h 10 % Tofu: 2 50 % Alcohol: 4	Pre-treatment by fermentation improved photosynthetic H_2 production		(Zhu et al. 1995)
Glucose	*C. butyricum* SC-E1 continuous	2.0-2.3 mol H_2/mol hexose	Sequential continuous	*Rhodobacter*	-	1.4-5.6 mol H_2/mol hexose (predicted) [a]	Hypothetical study	(Kataoka et al. 1997);
Starch + yeast extract + glutamate	*C. butyricum* batch	1.9 mol H_2/mol hexose [a]	Sequential batch-transfer	*Rhodobacter* sp. M-19 PNS bacteria	1.7 mol H_2/mol hexose 32.4 % efficiency [b]	mol H_2/mol hexose 3.6 [a]	Medium included glutamate	(Yokoi et al. 1998)
		-	Co-culture 1:10 (mass)		-	Batch: 4.5 [a] Repeated fed-batch: 6.4 [a]	Fed-batch performed for 30 days	
glucose	*C. butyricum* NCIB 9576 semi-continuous	1.29 mol H_2 / mol hexose [b]	Sequential batch-transfer	*R. sphaeroides* E151 Immobilised in hollow fibres Fed-batch	0.36 mol H_2/mol hexose [b]	1.64 mol H_2/mol hexose [b]	-	(Kim et al. 2001)
Rice-wine wastewater		1 L H_2/L wastewater in 18 h [a]			0.44 L H_2/ L broth/ day for 10 days [a]	1.44 H_2/L broth/ day [a]	-	
Tofu wastewater		0.9 L H_2/L wastewater in 26 h [a]			0.2 L H_2/ L broth/day for 30 days [a]	1.1 L H_2/L broth/day [a]	-	
Glucose	*C. butyricum* batch, immobilised	NG 62% of H_2	Immobilised co-culture *ca.* 1:1 (mass)	*R. sphaeroides* RV batch, immobilised	NG 38% of H_2	NG	H_2 produced for *ca.* 24 h	(Zhu et al. 2001)
Tofu wastewater		-			-	2.2 L H_2/L wastewater	H_2 produced for *ca.* 48 h	(Zhu et al. 2002)

Sweet potato starch residue + polypepton or corn steep liquor	*C. butyricum* & *Enterobacter aerogenes* co-culture initially *ca.* 2:1 (w:w) [b] Repeated-batch HRT: 2 d	2.7 mol H_2/mol hexose [a]	Sequential batch-transfer	*Rhodobacter* sp. M-19 + 20µg/l Na_2MoO_4 + 10 mg/l EDTA Repeated-batch HRT: 6.25 d	4.5 mol H_2/mol hexose [a]	7.2 mol H_2/mol hexose [a]	Performed for >30 days	(Yokoi et al. 2001; Yokoi et al. 2002)
Potato steam peel hydrolyzate	*Caldicellulosiruptor saccharolyticus* batch	2.9 mol H_2/mol hexose [b]	Sequential batch-transfer	*R. capsulatus* + yeast extract 31 ºC, Batch	45.6 % efficiency [b]	5.64 mol H_2/mol hexose [a]	Glucose was not the sole substrate in hydrolyzate	(Claassen et al. 2004)
Algal biomass (starch) *C. reinhardtii*	*Clostridium butyricum* batch	2.6 mol H_2/mol hexose [a]	Sequential batch-transfer	*R. sphaeroides* KD131 PNS bacteria + glutamate, batch	88 % efficiency [b]	8.3 mol H_2/mol hexose [a]	Starch was not the sole substrate in algal biomass	(Kim et al. 2006c)
Glucose	Anaerobic bacteria Continuous, 37 °C	1.36 mol H_2/mol hexose , + acetate, propionate, butyrate [a]	Sequential batch-transfer	*Rhodopseudomonas capsulata* 35 °C, continuous	3.2 mol H_2/mol hexose [a] 40 % efficiency	4.56 mol H_2/mol hexose [a]	Glutamate added to stage-1 effluent. 1st stage maintained for over 6 months, 2nd for over 10 days.	(Shi & Yu 2006)
Facultative aerobes/anaerobes – Anoxygenic photosynthetic bacteria								
Dextrose	*Streptococcus faecalis*	lactate (0.35 M) No H_2	Sequential batch-transfer	*Rhodospirillum rubrum* S-1 Fed-batch, 30 °C	99 % efficiency 16-24 mL H_2/g/h	NG	1st stage was industrial yoghurt production	(Zurrer & Bachofen 1979)
Cellulose	*Cellulomonas* sp. batch	Hexose → organic acids (no H_2)	Co-culture 1:1 (vol)	*Rhodopseudomonas. capsulata* B100 (WT) batch	-	1.2-4.3 mol H_2/mol hexose [a]	20 mL scale. All H_2 from 2nd stage. ST410 is a H_2 uptake deficient mutant	(Odom & Wall 1983)
				R. capsulata ST410 batch	-	4.6-6.2 mol H_2/mol hexose [a]		
Glucose	*Klebsiella pneumoniae* Continuous 18-19 ºC	NG	Immobilised co-culture (ratio NG)	*Rhodospirillum rubrum* Continuous 18-19 ºC	NG	1.3-5.3 mol H_2/mol hexose [b] 10 days	*K. pneumoniae* was a contaminant	(Weetall et al. 1989)
Sawdust hydrolysate						6.6-8.4 mol H_2/mol hexose [b] 30 days		
Cellulose hydrolysate						NG 46 days		

Molasses	NG Industrial lactic acid production	No H_2 3.4 mM lactate in wastewater	Sequential batch-transfer	*Rhodobacter sphaeroides* O.U.001 Batch, 30 ºC	> 100 % based on lactate content of wastewater	4480 mL H_2/L wastewater	Wastewater contained non-lactate substrates and was diluted 10-fold	(Sasikala et al. 1991)
algal biomass (starch) *C. reinhardtii*	Mixed bacterial community enriched on succinate	-	Co-culture	Consortium: *Rhodobium marinum*, *Vibrio fluvialis* and *Proteus vulgaris*	-	1.13 mol H_2/mol hexose [a]	Algal biomass may contain substrates other than starch	(Ike et al. 1997)
	Lactobacillus amylovorus Batch	Hexose → lactic acid 70-80% (no H_2)	Sequential batch-transfer	*Rhodobacter sphaeroides* RV batch + 10 mM glutamate	41.7 % efficiency [b]	4.6 mol H_2/mol hexose [a]		
Starch	*Vibrio fluvialis* Batch	Acetate & ethanol (no H_2)	Sequential batch-transfer	*Rhodobium marinum* A-501 (halophile)	100 % of H_2 95 % efficiency	2.4 mol H_2/mol hexose [b]	-	(Ike et al. 2001)
algal biomass (starch) *C. reinhardtii*	*L. amylovorus* batch	Lactate (no H_2)			100 % of H_2	7.9 mol H_2/mol hexose [a]	Starch was not the sole substrate in algal biomass	
	V. fluvialis	No H_2	Co-culture *ca.* 1:2 (mass)		100 % of H_2	6.2 mol H_2/mol hexose [a]		
algal biomass (starch) *C. reinhardtii* & *Dunaleilla tertiolecta*	*Lactobacillus amylovorus* batch, 30 ºC	No H_2 1.6 mol lactate/mol starch-hexose	Co-culture *ca.* 5:6 (mass)	*R. marinum* PNS bacteria batch, 30 ºC + 1.5 g/L $NaHCO_3$	100 % of H_2	7.3 mol H_2/mol hexose, 60.8 % [a]	stable pH, 13 days	(Kawaguchi et al. 2001)
			Sequential batch-transfer		3.4 mol H_2/mol lactose (57 %)	5.4 mol H_2/mol hexose, 45.3 % [a]	-	
Glucose	*Rhodopseudomonas palustris* P4 Dark-adapted	0.041 mol H_2 and 5.7 mol organic carbon/mol hexose [b]	Sequential batch-transfer	*Rhodopseudomonas palustris* P4 Light-adapted	10 % efficiency on fermentation broth	2-fold increase over dark fermentation alone [a]	Rate of H_2 photoproduction too low to be economically practical	(Oh et al. 2004)
Glucose	*Enterobacter cloacae* DM11 Batch, 37 ºC, static	1.86 mol H_2/mol hexose [a]	Sequential batch-transfer	*Rhodobacter sphaeroides* O.U.001 Batch, 30 ºC	37-43 % efficiency [a]	NG	-	(Nath et al. 2005)

	E. cloacae DM11 Batch, 37 ºC, stirred	3.31 mol H_2/mol hexose [a]		*R. sphaeroides* O.U.001 Batch, 34 ºC				(Nath et al. 2008)
Glucose	*Lactobacillus delbrueckii* Batch, 30 ºC	Lactate, acetate No H_2	Immobilised co-culture 4:11 (mass)	*R. sphaeroides* RV Batch, 30 ºC	100 % of H_2	7.1 mol H_2/mol hexose [a]	-	(Asada et al. 2006)
Glucose	*E. coli* HD701 Batch, 30 ºC	0.4 mol H_2/mol hexose	Sequential batch-transfer	*R. sphaeroides* O.U.001 Batch, 30 ºC	Acetate and ethanol consumed No H_2	0.4 mol H_2/mol hexose [a]	Inhibitory N-source in primary substrate	(Redwood & Macaskie 2006)
Glucose (60 mmol/day)	*E. coli* HD701 Continuous, 30 ºC HRT=30 days	1.6 mol H_2/mol hexose [a]	Sequential continuous transfer by electro-dialysis	*R. sphaeroides* O.U.001 Continuous, 30 ºC HRT=3 days	0.83 mol H_2/mol hexose 38 % efficiency	2.4 mol H_2/mol hexose [a]	Predicted yield: 10.1 mol H_2/mol hexose 2 stages not balanced	(Redwood & Macaskie 2007a, 2007b)
Non-axenic dark fermentation – Anoxygenic photosynthetic bacteria								
Cow manure	Mixed bacterial culture from digestor	H_2, CH_4, acetate, propionate, butyrate	Sequential batch-transfer	Mixed, predominantly *Rhodopseudomonas* spp.	10 g dw/L	Disposal of wastes & generation of biomass	Biomass produced rather than H_2	(Ensign 1977)
Poultry manure	Extant feed microbes	acetate, propionate, butyrate			11 g dw/L			
Palm oil mill effluent	Palm oil sludge	Main products: Acetate and propionate, no H_2, no NH_4^+	Sequential batch-transfer	*Rhodobacter sphaeroides* + NH_4Cl to 0.25 g/l continuous	No H_2 PHB	1 g PHB/l feed	Valuable alternative product	(Hassan et al. 1997)
Fruit & vegetable waste	Extant feed microorganisms, batch, ambient temperature	Main product: lactate, no H_2	Sequential batch-transfer	*Rhodobacter sphaeroides* RV + Mo, 30 ºC cont. chemostat	100 mL H_2/g dw/h for 10 days	NG	2^{nd} stage produced H_2 for 10 days, then switched to PHB	(Fascetti et al. 1998)
				R. sphaeroides RV WT, 30 ºC cont. chemostat	Max. 100 mL H_2 g dw/h (1^{st} 24 h)	NG	10 days H_2, then PHB	(Franchi et al. 2004)

				Strain SMV087 PHB$^-$, H_2 uptake$^-$ cont. chemostat		NG	> 45 days H_2	
Glucose UASB	NG anaerobic bacteria Batch, > 43 ºC	Main product: butyrate, no H_2	Sequential batch-transfer	Contents of 1st stage + *Rhodopseudomonas palustris* Batch, 35 ºC	Headspace gas 7 % H_2 4 % CH_4	NG	toxic products from 1st stage: H_2S and ethanol	(Lee et al. 2002)
Glucose CSTR					*ca.* 14 % H_2 *ca.* 2 % CH_4	NG		
Glucose & beef extract CSTR					*ca.* 55 % H_2 0 % CH_4	NG		
Olive Mill Wastewater (Diluted 50 %)	Acclimated sludge Batch, 30 ºC	No H_2	Sequential batch-transfer	*Rhodobacter sphaeroides* O.U.001 Batch, 30 ºC	100 % of H_2	29 L H_2/L feed	Pre-treatment of the feed lessened the need for dilution	(Eroğlu et al. 2006)
Sucrose	Cattle dung Batch, 38 ºC	1.29 mol H_2/mol hexose	Sequential batch-transfer	*R. sphaeroides* SH2C Batch, 30 ºC	63-70 % efficiency [a]	3.32 mol H_2/mol hexose [a]	-	(Tao et al. 2007)
Non-biological – Anoxygenic photosynthetic bacteria								
algal biomass (starch) *C. reinhardtii*	Heat-HCl treatment	Glucose, fatty acids and NH_4^+	Sequential batch-transfer	*R. sphaeroides* RV batch + 10 mM glutamate	0.02 mol H_2/mol hexose [a]	0.02 mol H_2/mol hexose [a]	-	(Ike et al. 1997)

Cultures contained free cells (and not immobilised) unless otherwise stated. NG: not given in source and/or cannot be calculated from given data. [a] Value given in original cited source. [b] Authors calculations from source data. *Thermophiles are classed tentatively as strict anaerobes; *Thermotoga* spp. may in fact be microaerophiles (Van Ooteghem et al. 2004). Accounts are sorted according to the type of organism used in the 1st stage and then by date, grouping work by the same authors). Some accounts have been omitted due to insufficient data.

Table 2 : Bottlenecks to the application of anoxygenic photosynthetic bacteria in H_2 production.

Limitation	Effect	Solutions	Progress	Ref
Low light conversion efficiency due to unsuitable light intensity	Large land area needed due to low intensity of solar illumination and shallow cultures	Develop strains with truncated light harvesting antenna	Proven at lab-scale	(Miyake et al. 1999; Vasilyeva et al. 1999; Kondo et al. 2002; Kim et al. 2004; Kondo et al. 2006; Kim et al. 2006a)
		Improved photobioreactor design	Ongoing	(Tsygankov 2001; Hoekema et al. 2002; Wakayama & Miyake 2002; Hoekema et al. 2006; Claassen & de Vrije 2007)
		Immobilisation; adaptation to a more constant light intensity	Proven at lab-scale	(Zhu et al. 2002; Gosse et al. 2007)
Sub-optimal conversion of substrates to H_2	Diversion of carbon, reductant and ATP into PHB synthesis detracts from H_2 production	Develop PHB deficient strains	Proven at pilot-scale	(Hustede et al. 1993; Lee et al. 2002; Franchi et al. 2004; Kim et al. 2006b)
Requirement for CO_2 (species- and substrate-dependent, see text)	Limited substrate uptake; continuous gas purging prevents cycling of produced CO_2	Recirculation of headspace gas	Proven at pilot-scale	(Hoekema et al. 2002)
		Use of species not requiring CO_2 e.g. *R. sphaeroides, R. capsulatus*	Proven at lab-scale	(Ivanovskii et al. 1997; Filatova et al. 2005a; Filatova et al. 2005b)
H_2 uptake detracts from net H_2 production	Decreased net H_2 production	Develop strains deficient in uptake Hydrogenases	Proven at pilot-scale	(Willison et al. 1984; Jahn et al. 1994; Worin et al. 1996; Lee et al. 2002; Öztürk et al. 2006; Kim et al. 2006b)
		Metal limitation (e.g. using EDTA) to prevent synthesis of active uptake hydrogenases	Proven at lab-scale	(Kern et al. 1992)
Culture contamination	Loss of PNS bacteria due to overgrowth of contaminants	Selective chemical inhibitors	Proven at lab-scale	(Liessens & Verstraete 1986)
		Blue light-filters prevent algal growth	Proven at lab-scale	(Ko & Noike 2002)
Nitrogenase 'switch-off' in response to fixed sources of N (esp. NH_4^+)	Limited to using substrates with high C/N ratio	Use of NH_4^+-insensitive strains (derepression of nitrogenase)	Proven at lab-scale	(Wall & Gest 1979; Zinchenko et al. 1991; Yagi et al. 1994; Zinchenko et al. 1997)
		Anion-selective immobilisation matrices	Proven at lab-scale	(Zhu et al. 1999b; Zhu et al. 2001)
		Electroseparation of NH_4^+	Proven at lab--scale	(Redwood & Macaskie 2007a)

Table 3 : Potential productivities of algal/cyanobacterial-driven dual systems

1st stage		Dual system yield (mol H_2/mol hexose)	Theoretical rate of H_2 production (mol H_2/m^2/day) *	Light capture area needed to power 1 home (m^2) **	Source
Organism	Photoautotrophic productivity (mol hexose/m^2/day)*				
Chlamydomonas sp.	NG Assume 0.158	8	1.27	451.7	(Miura et al. 1992)
Chlamydomonas sp.	Av. : 0.0244 Max. : 0.0926	5.8	Av. : 0.142 Max. : 0.537	Av. : 4039.4 min. : 1068.2	(Akano et al. 1996; Ikuta et al. 1997)
Synechoccus cedrorum	NG Assume 0.158	0.702 (free cells)	0.111	5167.6	(Sasikala et al. 1994a)
Spirulina platensis	NG Assume 0.158	2	0.317	1809.5	(Aoyama et al. 1997)
Clostridium butyricum	0.158 microalgal starch	8.3	1.315	436.2	(Kim et al. 2006c)
Lactobacillus amylovorus	NG Assume 0.158 cyanobacterial glycogen	4.6	0.729	786.8	(Shi & Yu 2006)
Lactobacillus amylovorus	NG Assume 0.158 microalgal starch	7.3 (co-culture)	1.157	495.8	(Ike et al. 2001)
		5.4 (sequential)	0.856	670.1	

NG: The productivity of carbohydrate accumulation was not given and could not be calculated from given data. The assumed value of 0.158 mol hexose/m^2/day was calculated from published data (Kim et al. 2006c). Photoautotrophic productivity was assumed to be similar after scale-up and under dual system conditions but may be less e.g. due to light limitation in co-culture.

* by multiplying the photoautotrophic productivity with the dual system yield.

** Assuming a home can be powered by a 1 kW PEM fuel cell demanding 23.9 mol H_2/h and operating at 50 % efficiency and 95 % H_2 utilisation (Levin et al. 2004a).

Values are authors' calculations from data given in the published sources shown.

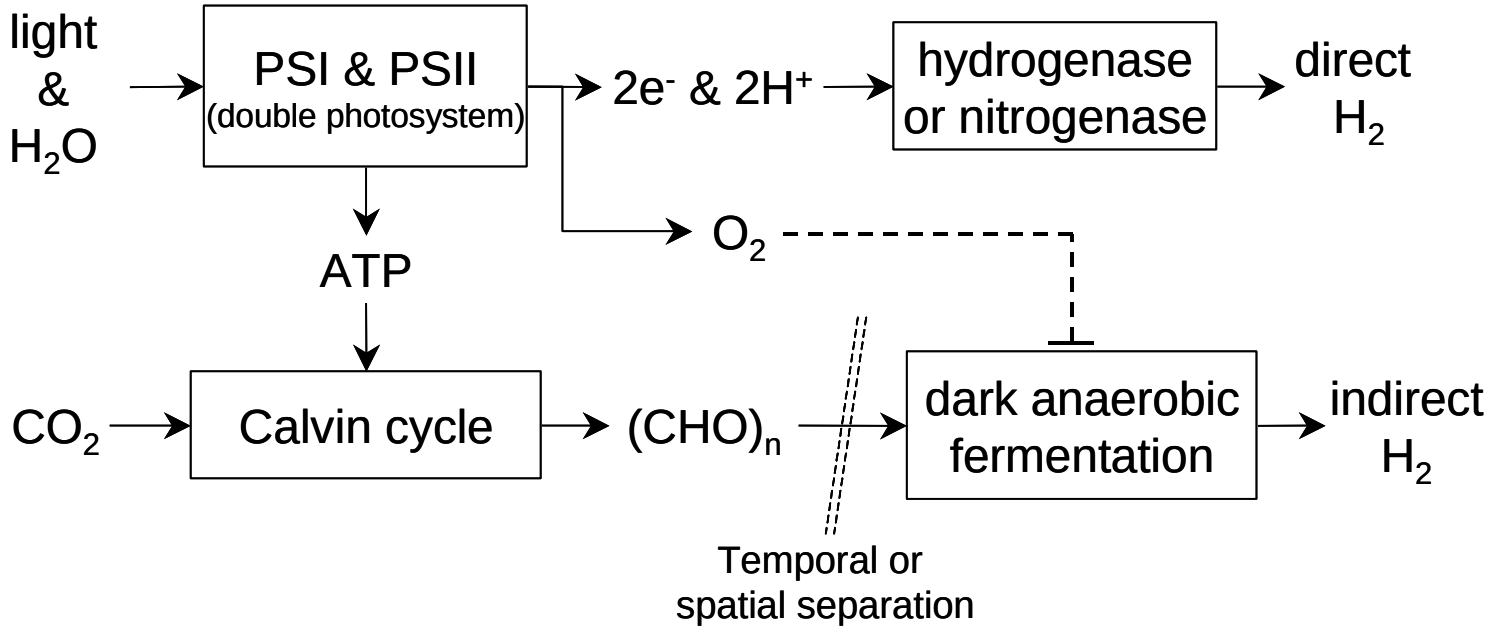
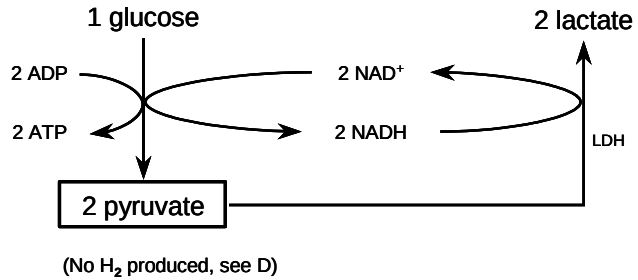
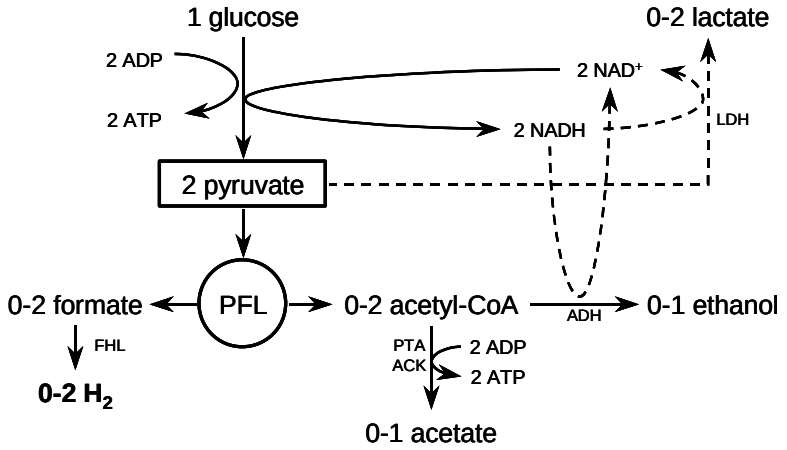


Figure 1: Direct and indirect photolysis. Through direct photolysis, the H_2 evolving enzyme is hydrogenase in microalgae and nitrogenase in cyanobacteria (see text). The dotted line represents the avoided inhibition of dark fermentation by O_2 *via* indirect photolysis.

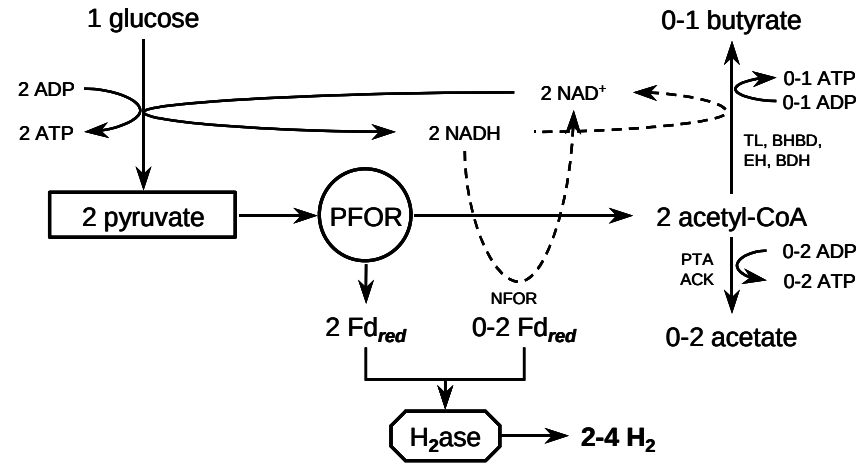
A: Lactic acid fermentation e.g. *Lactobacillus amylovorus*



B: Mixed-acid fermentation e.g. *Escherichia coli*



C: Anaerobic fermentation e.g. *Clostridium butyricum*



D: Effect of fermentation-type on hypothetical dual systems

Ideal fermentations	Ideal photo-fermentations
A: *L. amylovorus*	
1 glucose → 2 lactate	2 lactate → 12 H_2
0 H_2 + 12 H_2 = 12 H_2	
B: *E. coli*	
1 glucose → 2 H_2 + 1 ethanol + 1 acetate	1 ethanol + 1 acetate → 10 H_2
2 H_2 + 10 H_2 = 12 H_2	
C: *C. butyricum* (a mixture of two reactions)	
1 glucose → 4 H_2 + 2 acetate	2 acetate → 8 H_2
4 H_2 + 8 H_2 = 12 H_2	
1 glucose → 2 H_2 + 1 butyrate	1 butyrate → 10 H_2
2 H_2 + 10 H_2 = 12 H_2	

Figure 2: Suitable dark fermentations for dual systems. Pathways are abridged to highlight the overall balances. Dotted lines indicate alternative/competing pathways. Abbreviations: LDH lactate dehydrogenase, PFL pyruvate:formate lyase, ACK acetate kinase, FHL formate:hydrogen lyase, PTA phosphotransacetylase, ADH alcohol dehydrogenase, NFOR NADH:ferredoxin oxidoreductase, TL thiolase, BHBD hydroxybutyryl-CoA dehydrogenase, EH enoyl-CoA hydratase, BDH butyryl-CoA dehydrogenase. Compiled from Sode et al. (2001) and Chen et al. (2006).

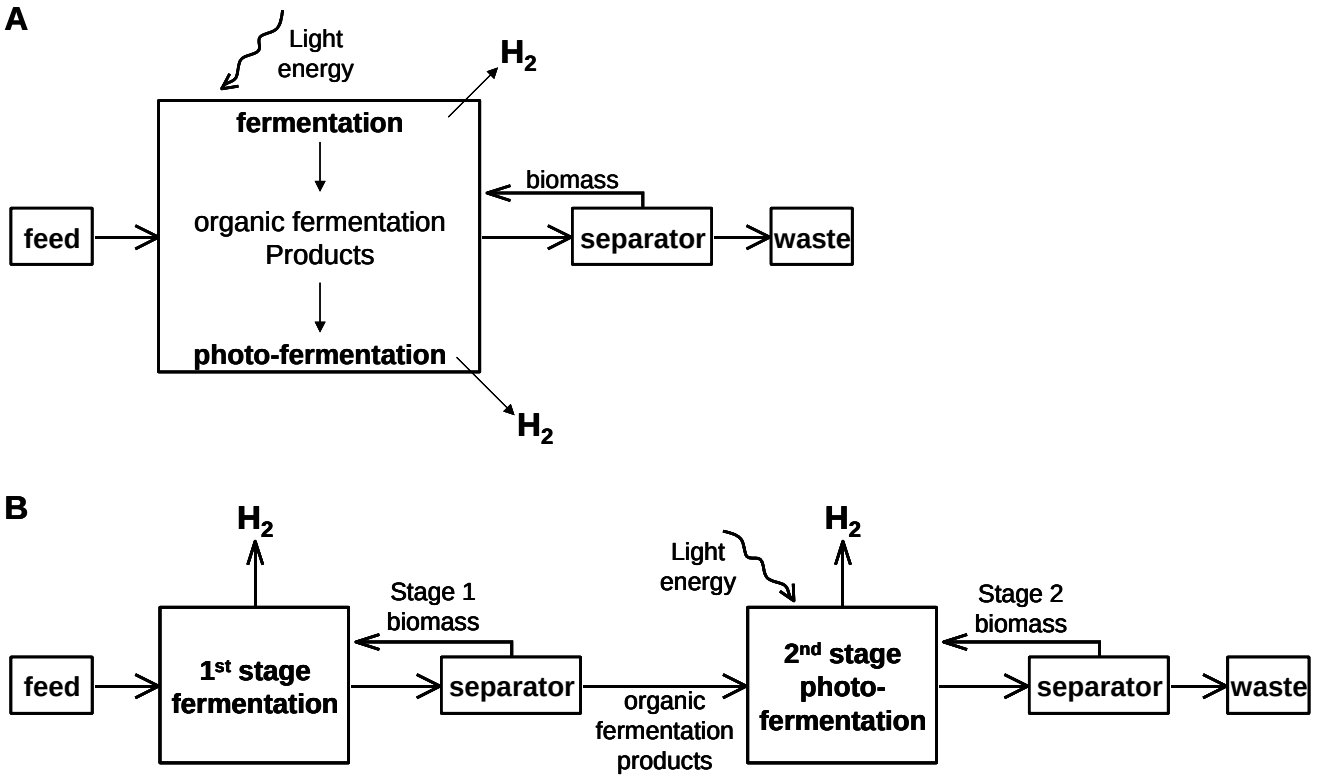


Figure 3: Dual systems in co-culture (A) and in sequential reactors (B).

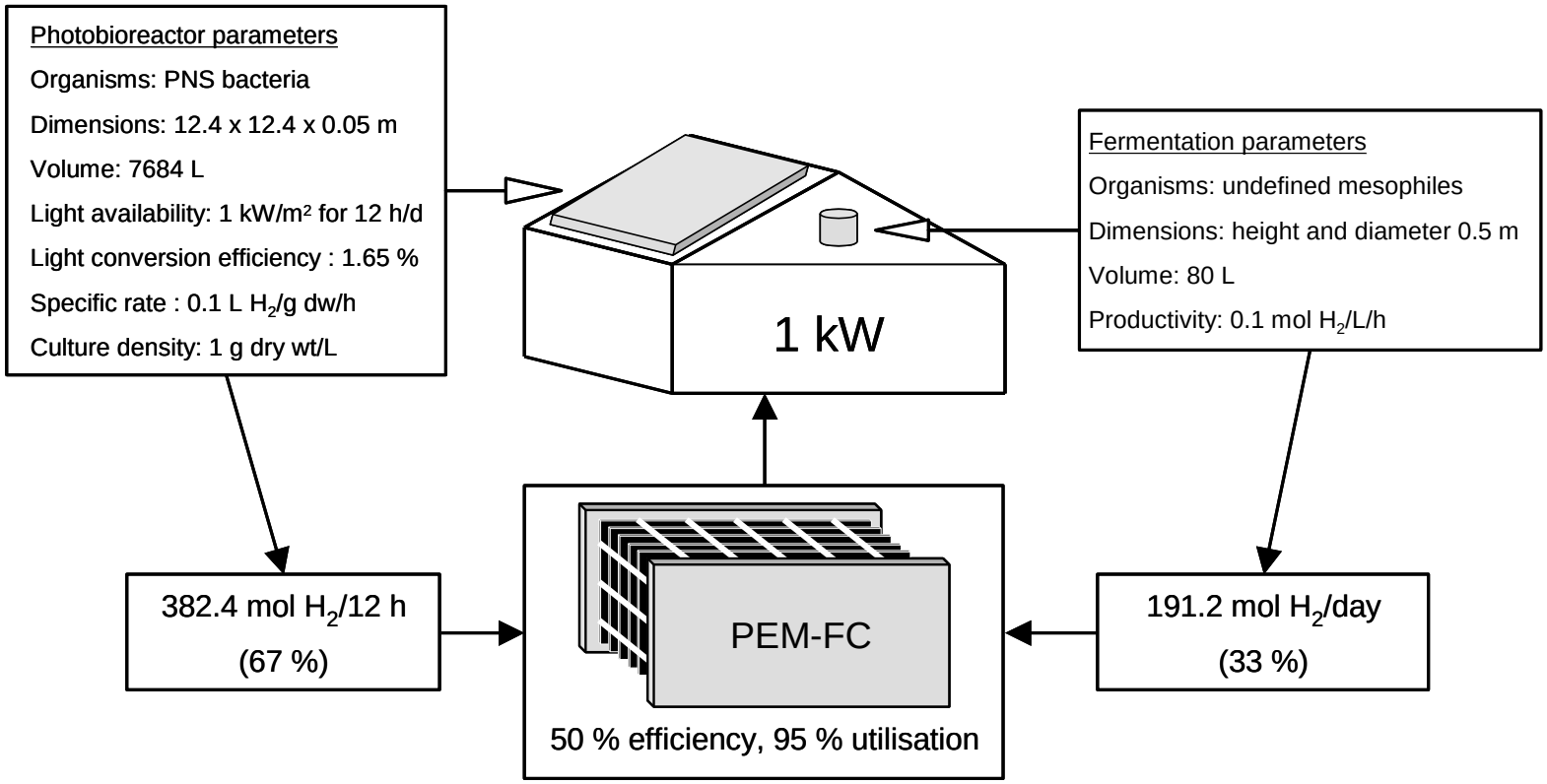


Figure 4: Spatial feasibility of de-centralized energy generation. The cartoon depicts one possible configuration of a sequential dual system combining dark fermentation and PNS bacteria. Detailed explanation is given in section 3.4.2.3.